Cell. Mol. Life Sci. 62 (2005) 2792–2810
1420-682X/05/232792-19
DOI 10.1007/s00018-005-5269-y

Cellular and Molecular Life Sciences

Review

Molybdenum cofactor biosynthesis and deficiency

G. Schwarz[+]

Institute of Plant Biology, Technical University Braunschweig, Spielmannstrasse 7, 38106 Braunschweig (Germany)

Received 17 June 2005; received after revision 18 August 2005; accepted 1 September 2005
Online First 2 November 2005

Abstract. The molybdenum cofactor (Moco) forms the active site of all molybdenum (Mo) enzymes, except nitrogenase. Mo enzymes catalyze important redox reactions in global metabolic cycles. Moco consists of Mo covalently bound to one or two dithiolates attached to a unique tricyclic pterin moiety commonly referred to as molybdopterin (MPT). Moco is synthesized by an ancient and conserved biosynthetic pathway that can be divided into four steps, according to the biosynthetic intermediates precursor Z (cyclic pyranopterin monophosphate), MPT and adenylated MPT. In a fifth step modifications such as attachment of nucleotides, sulfuration or bond formation between Mo and the protein result in different catalytic Mo centers. A defect in any of the steps of Moco biosynthesis results in the pleiotropic loss of all Mo enzyme activities. Human Moco deficiency is a hereditary metabolic disorder characterized by severe neurodegeneration resulting in early childhood death. Recently, a first substitution therapy was established.

Key words. Molybdenum cofactor; molybdopterin; cyclic pyranopterin monophosphate; copper; biosynthesis; deficiency; therapy.

Introduction

Molybdenum (Mo) is the only second row transition element that exhibits biological activity when it is ligated to a cofactor [1]. The vast majority of all Mo-dependent enzymes use the metal in the form of Mo cofactor (Moco), which consists of Mo covalently bound to the dithiolate moiety of a tricyclic pterin referred to as molybdopterin (MPT) [2]. The other type of Mo-containing cofactor is exclusively found in nitrogenase, forming the so-called iron-Mo cofactor (FeMoco) [3]. Both types of cofactors have in common that the metal is surrounded by sulfur and oxygen atoms. The main difference is that the Mo-binding sulfurs are either part of an ene-thiolate function (Moco) or positioned by a cluster of iron and sulfur atoms (FeMoco). The primary role of both cofactors is to control the redox properties of Mo via sulfur-mediated coordination. So far it is not fully understood how the redox state of the pterin moiety in Moco or the Fe-S cluster in FeMoco contributes to the metal redox behavior and electron transport from or to the metal site.

Moco-containing enzymes catalyze important redox reactions in the global carbon, sulfur and nitrogen cycles that are characterized by transfer of an oxygen atom, ultimately derived from or incorporated into water, to or from a substrate in a two-electron reaction [4]. They are classified into three families depending on the coordination chemistry of the Mo ligand [5]. In mammals, the most important Mo enzyme is sulfite oxidase (SO), which catalyzes the last step in the degradation of sulfur-containing amino acids and sulfatides [6]. Very similar to sulfite oxidase is eukaryotic nitrate reductase (NR), which is only found in autotrophic organisms, where it catalyzes the first and rate-limiting step in nitrate assimilation

[+] Present address: Institute of Biochemistry, University of Cologne, Otto-Fischer-Str. 12–14, 50674 Köln (Germany), Fax: +49 221 470 6731, e-mail: gschwarz@uni-koeln.de

[7]. Both enzymes make up the sulfite oxidase family of Mo enzymes. Xanthine oxidoreductases (XORs) form another class of Mo enzymes; they are involved in purine catabolism as well as cellular responses to senescence and apoptosis [8]. Aldehyde oxidases (AOs) belong to the same family and are important for phytohormone synthesis in plants [9]. The members of the dimethylsulfoxide (DMSO) reductase family are only found in prokaryotes and coordinate the metal by two equivalents of the pterin cofactor [10]. This bacterial form is found in a variety of Mo enzymes that are mainly used during anaerobic growth such as dissimilatory NR, formate dehydrogenase and trimethylamine N-oxide reductase with the corresponding substrates as terminal electron acceptor. With only the exception of carbon monoxide dehydrogenase, which is a binuclear Mo and copper-containing Fe-S flavoprotein [11], in all other known pro- and eukaryotic enzymes the cofactor is mononuclear. In order to complete the catalytic cycle, a reductive or oxidative half-reaction involves an intramolecular electron transport chain from or to the substrate using other prosthetic groups as cofactors and co-substrates, such as heme, Fe-S clusters, flavin adenine dinucleotide (FAD) or the reduced form of nicotinamide adenine dinucleotide (NADH), cytochrome c and oxygen.

Genetics of Moco

The investigation of Mo metabolism started with the genetic analysis of mutants of the filamentous fungus *Aspergillus nidulans* [12] that were defective in NR. Cove and Pateman [13] isolated NR-deficient mutants that showed a simultaneous loss of two Mo-dependent enzymes, NR and xanthine dehydrogenase (XDH). As Mo was the only link between those two – otherwise very different – enzymes, the authors suggested that both enzymes share a common Mo-related cofactor. In the following years, similar mutants were described for *Neurospora crassa* [14], *Escherichia coli* [15], *Drosophila melanogaster* [16], several higher plants [17] and humans [18]. As in all organisms up to six different genetic complementation groups have been identified, the existence of a multi-step biosynthetic Moco pathway was proposed.

Among eukaryotes, the molecular, biochemical and genetic analysis of Moco mutants was most advanced in the filamentous fungus *A. nidulans* and in higher plants. A mutational block of Moco biosynthesis leads to the loss of essential metabolic functions and can cause the death of the organism. In contrast to animals, Moco mutants of autotrophic organisms such as plants can be kept alive on media containing reduced nitrogen as N source, so that one can argue that the loss of NR activity is more dramatic for the plant than the lack of the other three Mo enzymes (XDH, AO, SO). Moco mutants have been described in numerous plants [17], but the phenotype of plant Moco mutants was best studied in *Nicotiana plumbaginifolia* [17], where all six Moco-specific genetic loci (*cnx*A–*cnx*F) showed a similar morphology strongly deviating from that of the wild type: stunted growth, chlorosis of leaves as well as small, narrow and crinkled leaves [19], which is probably caused by impairment of the plants to synthesize the phytohormones abscisic acid and indole acetic acid due to the loss of AO activities [9]. The consequences of Moco deficiency for humans will be discussed later.

Chemistry of Moco

Using the crude protein extract of the *Neurospora crassa nit-1* mutant [20], Nason and co-workers [21–23] provided first biochemical evidence for the existence of a cofactor common to all Mo enzymes. They were able to reconstitute the activity of inactive apo-NR by low molecular weight fractions derived from denatured Mo enyzmes of mammalian, plant and bacterial origin demonstrating the incorporation of a ubiquitous and universal cofactor. Consequently, nitrogenase as the only Mo-dependent enzyme that contains no Moco did not release a *nit-1* positive activity [24] because of its unique FeMoco. The observed complementation of *nit-1* apo-NR by Moco from different sources served as the basis for developing a sensitive biological in vitro assay referred to as the *nit-1* assay for cofactor determination.

Nason et al. [23] found that the reconstituting activity released from diverse Mo enzymes was very labile, with a lifetime of a few minutes, indicating the oxygen-sensitive nature of Moco. The need to include an excess of molybdate [25–27] into the reconstitution mixture indicated a strong tendency of Moco to lose Mo, while sulfhydryl reactive agents totally inhibited Moco activity in the *nit-1* assay [26–29], thus pointing to the participation of sulfur in metal coordination. The elucidation of the chemical nature of Moco is mainly based on the pioneering work of Rajagopalan and co-workers [2]. Due to the labile nature of Moco and its high sensitivity to air oxidation, most of the work was done by using degradation or oxidation products of Moco. Two oxidation products revealed the pterin nature of Moco and its C6 substitution with a unique four-carbon side chain [30]. A structural similarity between urothione, an excretion product in human urine, and the air oxidation product of Moco [31], the presence of sulfhydryl groups [28] and carbamidomethylation of Moco [32] demonstrated the coordination of the metal by a dithiolate group. The redox state of the pterin was proposed to be tetrahydro (fully reduced) [2, 32]. Crystal structures of different Mo enzymes confirmed the core structure of Moco and helped to clarify

the redox state of Moco. Due to the formation of a third pyrano ring between the C3' hydroxy group and the pterin C7 atom, a fully reduced hydrogenated pterin (tetrahydro state) is formed, which formally resembles a dihydropterin because it can be fully oxidized by two electrons [33]. The same type of metal binding pterin (MPT) is also involved in the coordination of tungsten in tungsten-containing bacterial enzymes that are structural and functional counterparts to their corresponding Mo enzymes [4].

Bacterial and eukaryotic Moco biosynthesis

The identification of several genetic complementation groups among Moco-deficient mutants in a given organism and the conserved structure of Moco provided a basis to propose an evolutionarily old multi-step biosynthetic pathway [34]. Already in pre-genomic times, a detailed mutant characterization contributed substantially to our understanding of the genetics and biochemistry of Moco biosynthesis in bacteria, plants, fungi and even humans. Investigations such as phenotype suppression by external molybdate or reconstitution experiments mixing cell-free protein extracts of different complementation groups provided evidence for two intermediates of the biosynthetic pathway. Furthermore, defects in molybdate uptake and processing could be assigned to specific mutants. A substantial part of our knowledge about Moco biosynthesis has been obtained from studies in *E. coli,* for which five Moco specific operons are known, designated *moa*, *mob*, *mod*, *moe* and *mog* and comprising more than 15 genes [35]. The first model of Moco biosynthesis was presented by Rajagopalan and co-workers [2]. Together with studies of eukaryotic Moco biosynthesis, during the last years a more complex picture of this pathway has been obtained [36].

In all organisms studied so far, Moco is synthesized by a conserved biosynthetic pathway that can be divided into five steps, according to the biosynthetic intermediates precursor Z, MPT, adenlyated MPT and Moco (fig. 1). At least six gene products catalyzing Moco biosynthesis have been identified in bacteria [2], fungi [37–40], plants [36] and humans [41–43]. Similar to the biosynthesis of other pterins and flavines, Moco synthesis starts with GTP [44], which is converted into the first intermediate, precursor Z. GTP conversion is catalyzed by the action of two proteins, one of them belonging to the family of radical S-adenosylmethionine-dependent enzymes that contain oxygen-sensitive Fe-S clusters [44, 45]. In the second step two sulfur atoms are transferred by the heterotetrameric enzyme MPT synthase [46], thus yielding MPT with the characteristic dithiolate function important for metal chelation. Each of the MPT sulfurs derives from one of the two small subunits of MPT synthase that carry a C-terminal thiocarboxylate [47]. After each reaction MPT synthase has to be regenerated by an ATP-dependent sulfur transfer reaction [48–50], yielding thiocarboxylated small subunits. In the next step MPT is further processed by adenylation, thus forming adenylated MPT [51]. In the final and most diverse steps of Moco biosynthesis Mo is attached to one (in pro- and eukaryotes) or two MPT dithiolates (in prokaryotes). Metal insertion is tightly coupled to MPT-AMP hydrolysis [52]. In many bacteria (*E. coli*), Mo insertion is tightly coupled to a fifth step that involves nucleotide attachment forming bis-MPT guanine or mono-MPT cytosine dinucleotide (MGD or MCD) [53, 54]. The transfer of a third (terminal) sulfur atom to Moco generates the cofactor form that is found in enzymes of the XOR family and can be considered as fifth step in pro- and eukaryotic Moco synthesis [55, 56]. Maturation of the dinuclear CuSMo center in carbon monoxide dehydrogenase represents a similar final modification [11]. In enzymes of the SO family a third sulfur ligand is derived from a cysteine residue of the apo-protein and occupies a similar position as the terminal sulfur in the XOR family [6, 57].

After completion of biosynthesis, the mature cofactor must be inserted into the Mo enzymes. In prokaryotes a complex of proteins synthesizing the last step(s) of Moco biosynthesis is proposed to donate the mature cofactor to the appropriate apo-enzymes [58], assisted by enzyme-specific chaperones [59]. In eukaryotes, the green alga *Chlamydomonas reinhardtii* is known to have a carrier protein that binds Moco and transfers it to NR [60].

With recombinant protein expression technology sufficient amounts of purified Moco synthetic proteins became available, which is a prerequisite for structural studies using X-ray crystallography. In the past 5 years Schindelin and co-workers [44, 46, 48, 61–64] substantially contributed to our current knowledge about the structures of bacterial Moco-synthetic proteins (fig. 2). In total, structures have been determined for seven proteins and protein complexes, though detailed knowledge is still missing about the catalytic mechanism of some proteins. However, the structures of MPT synthase and MoeB with MoaD provided important insights into the sulfur transfer mechanism during Moco biosynthesis.

Step 1: conversion of GTP into precursor Z

MPT is the only four-carbon side chain-substituted pterin known so far, while several other pteridines, such a biopterin, have three-carbon side chains [65]. Two major pathways are known for the synthesis of pteridines [65] and flavines [66] that start with the conversion of GTP by the enzymes cyclohydrolyse I and II, respectively. Based on labeling studies in *E. coli* [67], a complex reaction sequence [67] starting from GTP [44] generates the first

stable intermediate of the pathway, precursor Z. This pathway is different from the other two pteridine biosynthetic pathways, as the C8 atom of the purine base is inserted between the 2' and 3' ribose carbon atoms, thus forming the four-carbon atoms of the pyrano ring that is typical for Moco. Using ^{1}H nuclear magnetic resonance (NMR) studies with multiple labeled compounds, Rieder et al. [68] confirmed that each carbon atom of the ribose and the ring carbons of the guanine are incorporated into precursor Z. A model favored by two labs [67, 68] involves a ring-opening reaction followed by the subsequent protein-assisted transfer of the C8 formyl group

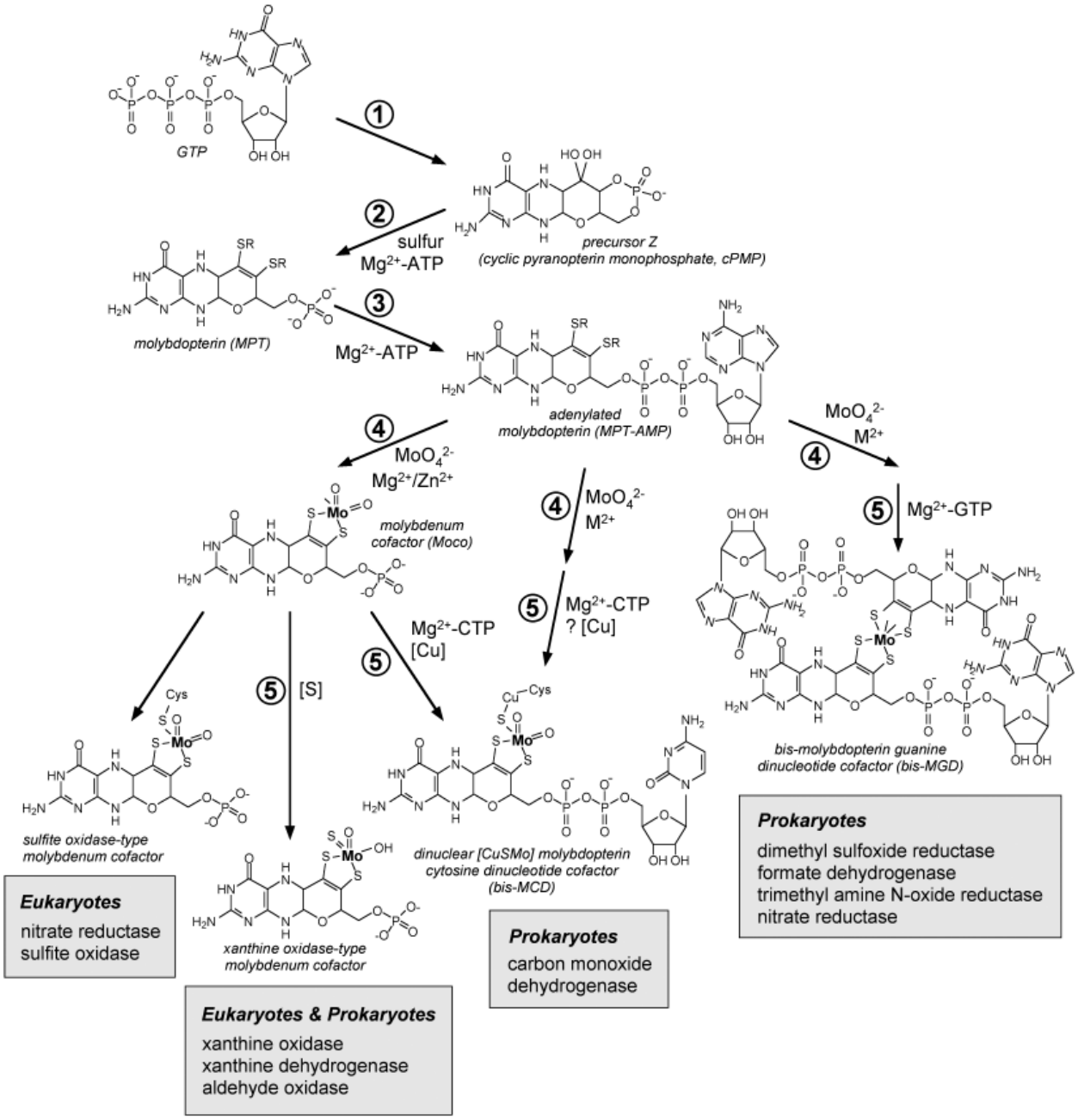

Figure 1. Biosynthesis and function of the Moco in bacteria and eukaryotes. Shown are the known biosynthetic intermediates of prokaryotic and eukaryotic Moco biosynthesis. Moco synthesis can be divided into five steps. For MPT and MPT-AMP, the ligands of the dithiolate sulfurs are indicated by an 'R', as it is currently unknown in which state copper is bound to the dithiolate. Upon Mo insertion it is also not clear how many oxo ligands are bound to the metal. Therefore, two Mo-oxo ligands are depicted, and a third line indicates an additional ligand. As it is unknown which divalent cation is used for MPT-AMP hydrolysis in prokaryotes, it is only shown as M^{2+}. For the synthesis of the CuSMo-bis-MCD cofactor, different routes of metal center assembly are possible, as indicated by the different arrows. After the fifth maturation step four different forms of Moco are generated that are found in different classes of Mo enzymes. Representative members are listed below.

that might be protein-bound via a thioester bond and could participate in a benzylic-acid-type rearrangement of the side chain [55], resulting in the C6-substituted four-carbon pterin precursor Z carrying a cyclic phosphate at C2' and C4' (fig. 3).

In *E. coli*, proteins MoaA and MoaC (fig. 2, table 1) were identified as essential for precursor Z synthesis. MoaA contains Fe-S clusters that are bound via highly conserved cysteine residues [69] and shows sequence similarities to a variety of proteins, including biotin synthase, pyruvate formate lyase and anaerobic ribonucleotide reductase [70]. MoaA and all homologues belong to the family of S-adenosylmethionine (SAM)-dependent radical enzymes. Members of this large family catalyze the formation of protein and/or substrate radicals by reductive cleavage of SAM by a [4Fe-4S] cluster [71]. Functional characterization of proteins involved in the first step of Moco biosynthesis started with the analysis of the human homologues MOCS1A (MoaA) and MOCS1B (MoaC) [72] (table 1). The *MOCS1* gene revealed two open reading frames separated by 15 nucleotides encoding the hypothetical proteins MOCS1A and MOCS1B [41]. Consequently, a bicistronic expression was proposed but could not be confirmed by expression analysis [72]. Only MOCS1A was translated from that bicistronic transcript. Analysis of *mocs1* genes and their expression in several vertebrates and invertebrates [73] showed a well-conserved novel messenger RNA (mRNA) splicing phenomenon in addition to the bicistronic transcript that also produces several classes of monocistronic transcripts encoding multi-domain proteins (MOCS1A-MOCS1B) [73].

Recombinant expression of both MOCS1A and MOCS1B in *E. coli moaA* and *moaC* mutants, respectively, demonstrated the functional conservation between bacterial and human Moco synthesis. Interestingly, MoaA and homologous proteins contain a conserved C-terminal double-glycine motif. Deletion or mutations in this motif resulted in the loss of MOCS1A function, indicating a crucial role in the catalytic mechanism [72]. As this motif is C-terminally blocked or deleted in splice variants that express MOCS1A-MOCS1B fusion proteins, the expression of several splice variants is needed for the activity of both *MOCS1* gene products.

Structures of both proteins essential for precursor Z synthesis have been determined from bacterial sources [44, 62]. The structure of MoaA from *Staphylococcus aureus* was determined in the apo-state as well as in complex with the co-substrate SAM [44]. The core of the protein is characterized by an incomplete triosephosphate isomerase barrel, which binds an N-terminal [4Fe-4S] cluster typical for SAM-dependent radical enzymes. The lateral opening of the incomplete barrel is covered by the C-terminal part containing a second, MoaA-specific [4Fe-4S] cluster. Both [4Fe-4S] clusters are extremely oxygen sensitive [45]; each one is coordinated by three cysteine residues. The fourth ligand of the N-terminal cluster is SAM, while the substrate 5'-GTP is believed to coordinate one non-ligated Fe in the C-terminal cluster. The function of MoaC, the other protein essential for precursor Z, is still enigmatic. MoaC forms a homohexamer

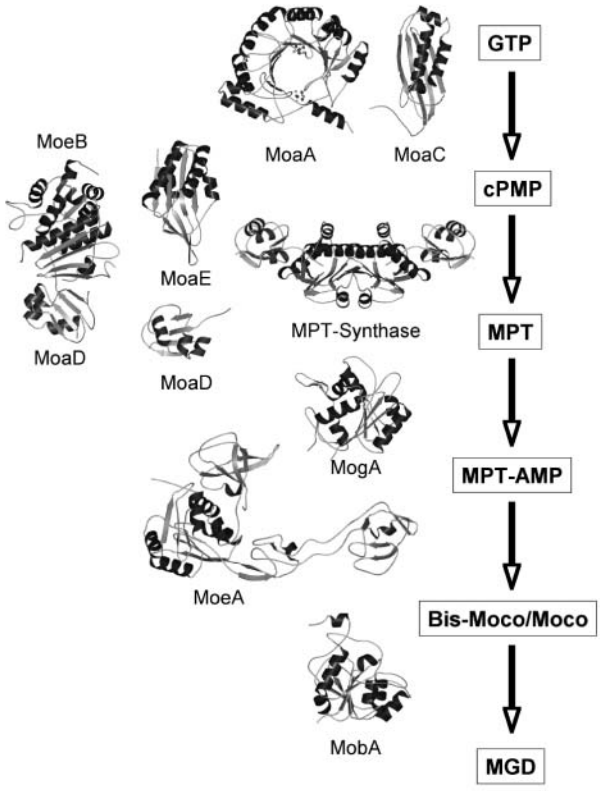

Figure 2. Crystal structures of bacterial Moco synthetic proteins. Ribbon presentation of crystal structures of bacterial Moco synthetic proteins MoaA [44], MoaC [62], MoaD, MoaE, MoeB-MoaD complex [63], MPT synthase [46], MogA [61], MoeA [64] and MobA [63]. α-Helices are shown in dark red or blue; β-strands are depicted in orange or light blue. Individual figures were generated with MOLSCRIPT [154] using the deposited coordinates from the protein structure data base and rendered with POVRAY (www.povray.org).

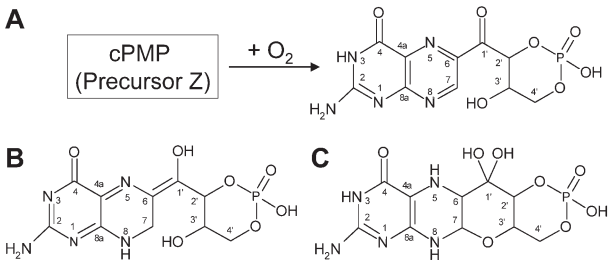

Figure 3. Structure of precursor Z/cyclic pyranopterin monophosphate (cPMP). (A) Oxidation of precursor Z/cPMP to compound Z. (B, C) Structures of precursor Z according to Wuebbens et al., 1993 [76] (B) and Santamaria et al., 2004 [83] (C).

Table 1. Comparison of proteins involved in bacterial and eukaryotic Moco biosynthesis.

Step	Bacteria *E. coli*	Fungi *A. nidualns*	Plants *A. thaliana*	Humans *H. sapiens*
1	MoaA [44, 67][c] MoaC [62, 67]	CnxA [37] CnxC [37]	Cnx2 [74] Cnx3 [74]	MOCS1A [41] MOCS1B [41]
2	MoaD [48, 86] MoaE [46, 86] MoeB [48, 155]	CnxG [39] CnxH [39] CnxF [38]	Cnx7 [156] Cnx6 [157] Cnx5 [94]	MOCS2A [42] MOCS2B [42] MOCS3 [158]
3[a]	MogA [61, 103] MoaB [70, 159]	CnxE (N) [40]	Cnx1 (C) [106, 160]	Geph. (N) [43, 106]
4[a]	MoeA [64, 109]	CnxE (C) [40]	Cnx1 (N) [160]	Geph. (C) [43, 161]
5[b]	MobA [63, 119, 126] MobB [162]	–	–	–
5[b]		HxB [132]	ABA3 [56]	HMCS [130]

[a] For two-domain proteins, the position of the domain is given in brackets (N, N-terminus; C, C-terminus).
[b] Step 5 comprises different modifications of the cofactor. Homologous proteins are shown in the same row.
[c] The second reference cites the structure if available.

[62] with a hypothetical active site formed by residues of two MoaC monomers.

The plant counterparts to MoaA and MoaC are Cnx2 and Cnx3, respectively (table 1). Both genes were cloned by functional complementation of the *E. coli* Moco mutants *moaA* and *moaC* [74], respectively, confirming the high degree of conservation in Moco biosynthesis. A similar situation to the human proteins in terms of expression pattern is seen in the fungus *A. nidulans* (CnxA and CnxC; [37, 75]).

Chemical properties and structure of precursor Z

Precursor Z was initially isolated and structurally characterized 12 years ago [76]. First the structure of compound Z [77], the air oxidation product of precursor Z (fig. 3A), was elucidated, and later this structure together with spectroscopic studies served as a basis to propose a structure for native precursor Z. In its first description, precursor Z was presented as quinoidal dihydropterin with a cyclic phosphate (fig. 3B). It was further reported that precursor Z was relatively stable against oxygen at low pH with an estimated half-life of several hours [76].

With the clarification of the Moco structure by crystal structures of different bacterial and eukaryotic Mo enzymes, it remained unclear at which stage the formation of the pyrano ring occurs. For XDH a ring closure during Moco insertion has been proposed [78], and in the crystal structure of bacterial NR, one of the two MPT equivalents has been observed in the ring-opened bicyclic form [79], indicating a possible role of the pterin redox state in catalysis. The proposed dihydro state of precursor Z was based on its two-electron oxidation to compound Z [76]. In contrast to precursor Z, dihydropterins are characterized by a high sensitivity to oxygen, with half-lives of minutes [80]. Tetrahydropterins such as tetrahydrobipterin are more stable at physiological pH ($t_{1/2}$ = 10–20 min) [81]. A recent study with different synthetic tetrahydro- and pyranopterins revealed a remarkable increase in the stability of pyranopterins in comparison with tetrahydropterins [82], which would fit much better with the observed stability of precursor Z.

The chemical structure of precursor Z was clarified after a fermentation and purification procedure has been developed resulting in sufficient amounts of precursor Z for detailed spectroscopic analyses [83]. Mass spectrometry and ^{1}H NMR spectroscopy demonstrated that precursor Z is indeed a pyranopterin, similar to Moco, and carries a geminal diol in the C1' position of the side chain. Therefore, precursor Z should be renamed 1',1'-dihydroxy 2', 4' cyclic pyranopterin monophosphate according to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature. For simplification, cyclic pyranopterin monophosphate (cPMP) will be used. The pyranopterin structure fits well with the two-electron oxidation of cPMP to compound Z and demonstrates that Moco is synthesized as pyranopterin. The 2geminal diol might have an additional protective function for cPMP and could also drive the subsequent sulfur transfer reaction in a particular direction. As geminal diols are only observed in a strong electrophilic environment, opening of either the pyrano- or cyclic phosphate should result in a loss of the hydration at C1' (see below).

Step 2: synthesis of the MPT dithiolate

In order to form the MPT dithiolate, two sulfur atoms must be incorporated into cPMP during the second step of Moco biosynthesis. This reaction is catalyzed by MPT synthase, a heterotetrameric complex of two small and two large subunits that stoichiometrically converts cPMP into MPT. MPT synthase activity was first identified in *E. coli* [84] using the *N. crassa nit-1* extract, which accumulates cPMP converted by an activity present in extracts of *E. coli moaA* (*chlA1*) mutants. *E. coli* MPT synthase is encoded by the *moa* operon, with MoaD (9 kDa) forming the small and MoaE (17 kDa) the large subunit [85]. Homologous proteins have been identified in fungi [39], humans [42] and plants [36] (table 1).

Purified MPT synthase and cPMP are sufficient to generate MPT in vitro [86]. Under conditions of cPMP excess, the formation of MPT was stoichiometric in respect to MPT synthase. Pitterle and Rajagopalan [85] found a 16-Da mass difference between MoaD proteins derived from active and inactive MPT synthase, pointing to exchange of an SH to an OH group. MoaD and homologous proteins are only conserved in their C-terminal region, which includes a double-glycine motif also found in ubiquitin, a crucial protein in eukaryotic protein degradation [87]. Direct biochemical studies using in vitro assembled MPT synthase from individually expressed and purified subunits demonstrated that the C-terminal end of MoaD carries the sulfur as thiocarboxylate [47]. The functional importance of the MoaD thiocarboxylate was also demonstrated by the crystal structure of *E. coli* MPT synthase, which shows that the C-terminus of MoaD is deeply inserted into the large subunit to form the active site [46]. The tetramer is formed by dimerization of two large subunits resulting in an elongated protein complex with two clearly separated active sites (fig. 2). Structural comparison between MoaD and ubiquitin shows a remarkable high degree of conservation [46].

Regarding the reaction mechanism of MPT synthase, two different theories were postulated in the past [47, 88] (fig. 4). Both models have in common that a mono-sulfurated reaction intermediate is formed, as each MoaD subunit carries only one sulfur atom. Gutzke et al. suggested the first S transfer at the C1' carbon of cPMP based on a structural proposal of cPMP that carried a keto function in the C1' [47]. Later Wuebbens and Rajagopalan [88] were able to isolate a mono-sulfurated intermediate with a terminal phosphate similar to MPT, pointing to an initial S transfer at C2'. In light of the refined cPMP structure carrying a geminal diol at C1' [83], a primary attack at C2' accompanied with an opening of the cyclic phosphate strengthens the latter model. Both theories differ in the mechanism by which the intermediate is converted into MPT. Gutzke et al. [47] propose a conformational change upon intermediate formation that results in its dissociation and increased binding to the second active site of the heterotetrameric enzyme (fig. 4A), where the second half of the reaction is completed. This model fits well with the

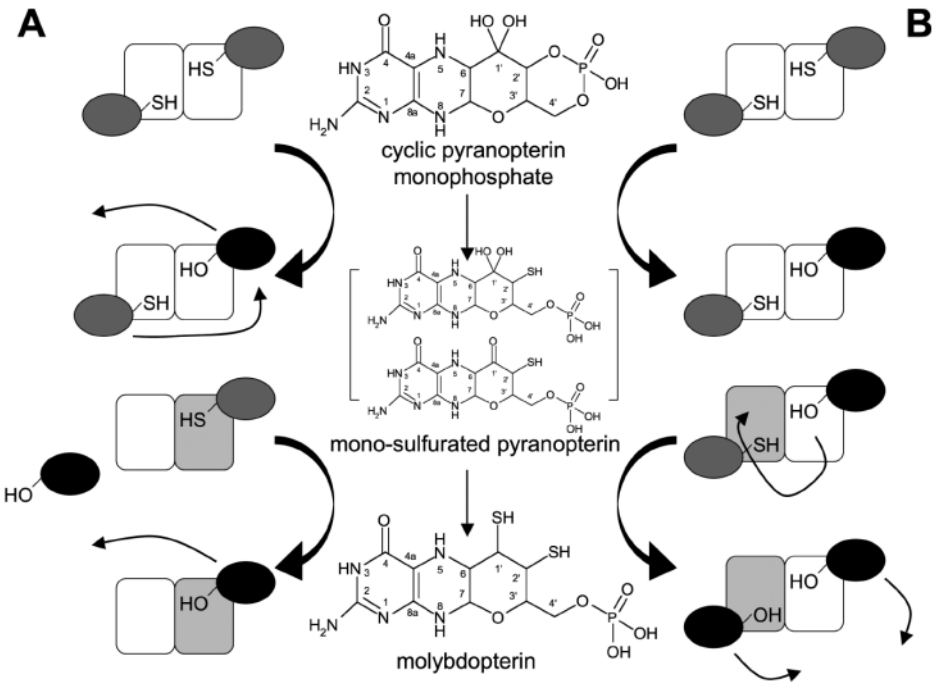

Figure 4. Mechanisms of MPT dithiolate synthesis. During MPT synthesis two sulfur atoms are transferred in two consecutive reactions. Both sulfurs are derived from two thiocarboxylated small subunits [47]. A mono-sulfurated pterin species is shown according to mechanistic [88] and structural studies [83]. Different theories were proposed to explain the stepwise transfer of sulfur from MPT synthase to cPMP. (A) The subunit exchange theory [88] is based on the dissociation of the first small subunit after intermediate formation (which stays bound) and binding of a second thiocarboxylated subunit. (B) The intermediate transfer hypothesis [47] is based on a conformational change upon intermediate formation and a subsequent transfer of the intermediate to the other active site carrying the second thiocarboxylated small subunit. The different shades of the small subunit illustrate active (gray) and inactive conformations (black). A conformational change in the large subunit is indicated by a light gray shade. LS, large subunit; SS, small subunit.

stoichiometric conversion of cPMP to MPT using purified MPT synthase. The other model [88] favors the release of the first small subunit upon sulfur transfer due to a tight binding of the mono-sulfurated intermediate (fig. 4B). In the second half-reaction a new thiocarboxylated small subunit binds to the intermediate-containing MoaE site and completes the reaction, which suggests that purified MPT synthase is disassembled after the first half-reaction and the second thiocarboxylated MoaD molecule has to move from one site to the other. Furthermore, excess of the thiocarboxylated small subunit should result in a stoichiometric turnover coupled to the amount of MoaD used, which has not been observed so far. Therefore, future experiments need to be done in order to uncover the molecular mechanism of MPT synthesis.

In plants, cloning of the small subunit of MPT synthase was achieved in a yeast two–hybrid screen using the assumed protein-protein interaction between both subunits [47]. In contrast to the first step in Moco synthesis, the approach of functional reconstitution was not successful in cloning the genes involved in the second step. Human MPT synthase is encoded by the *MOCS2* locus as bicistronic gene [42] similar to *MOCS1*. However, here bicistronic expression was demonstrated where the second reading frame (large subunit MOCS2B) is translated by a leaky scanning mechanism [42]. Applying the same method used before to identify the thiocarboxylate on bacterial MPT synthase resulted in in vitro formation of active human MPT synthase [89]. This system was subsequently used to investigate the molecular effect of mutations identified in human patients with Moco deficiency. Disease-causing mutations were due to a loss of the assembly of both subunits, reduced reaction rates or loss of substrate binding [89].

The source of the dithiolate sulfurs

In a separate reaction, sulfur is transferred to the small subunit of MPT synthase, which is catalyzed by one or more proteins (fig. 5). Bacterial MoeB is crucial for the activity of MPT synthase as *moeB* mutants accumulate a MoaD protein, which is free of sulfur and therefore 16 Da smaller than active MoaD [86]. The structural and functional similarity of MPT synthesis to ubiquitin-dependent protein degradation is not only restricted to MoaD. MoeB is homologous in its entire region to the N-terminal part of the ubiquitin-activating enzyme UbA1 from *Saccharomyces cerevisiae* [90, 91]. As UbA1 catalyzes the adenylation of ubiquitin, a similar role of MoeB was proposed. Further similarity between Moco and thiamin biosynthesis [92] supported a conserved mechanism of sulfur transfer in both pathways.

E. coli MoeB forms dimers and is able to interact with MoaD (fig. 2). Like UbA1, MoeB also catalyzes the adenylation of its target protein, MoaD, by using Mg-ATP as substrate [49] (fig. 5). Crystal structures of MoeB in complex with MoaD have been determined in its apo, ATP-bound and MoaD-adenylate forms [48], presenting a conserved mechanism of the acyl-adenylate formation in ubiquitin-dependent protein degradation, and in the synthesis of thiamin and Moco. In vitro, MoeB alone

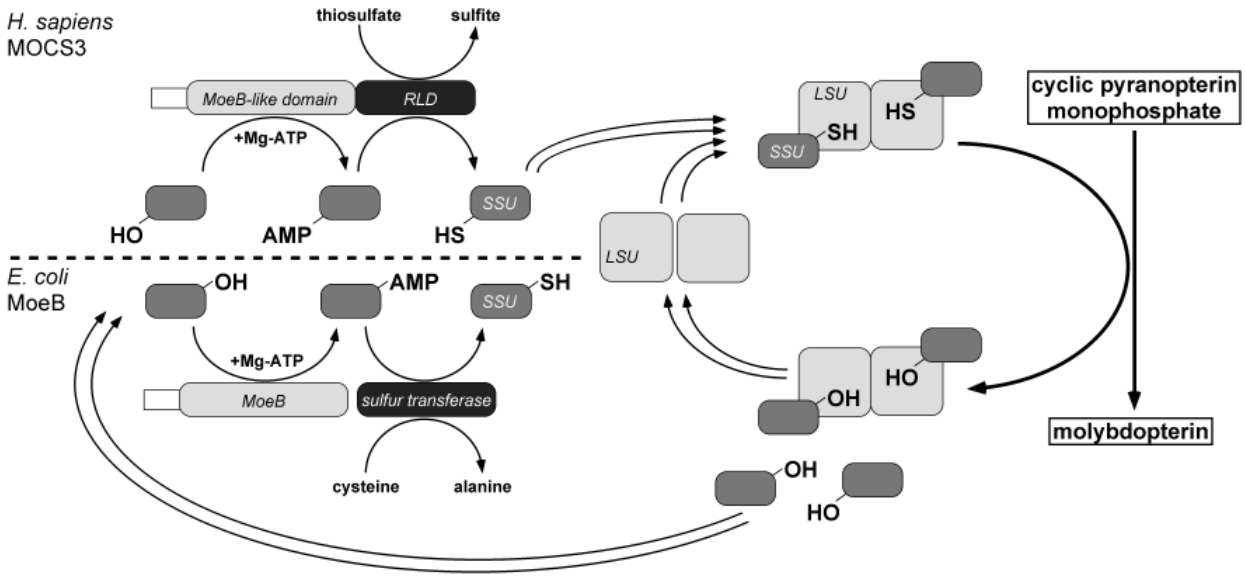

Figure 5. Sulfur transfer mechanism to MPT synthase. Thiocarboxylation of the small subunits of MPT synthase (SSU) is catalyzed by MoeB-like proteins. First an acyl adenylate is formed at the C-terminus of SSU catalyzed by MoeB (*E. coli*) or the MoeB-like domain in MOCS3 (humans). Activated SSU is subsequently sulfurylated in *E. coli* by transfer of a persulfide-bound sulfur from a not yet identified sulfur transferase [50], which is generated from the desulfuration of cysteine to alanine. In humans, the C-terminal rhodanese-like domain (RLD) of MOCS3 transfers a cysteine-bound persulfide, which can be generated by the desulfuration of thiosulfate in vitro [93, 95].

is not sufficient to reactivate carboxylated MoaD. Free sulfide or persulfide-loaded cysteine desulfurases such as IscS, CSD or CsdB/SufS are needed (fig. 5) to cleave the acyl-adenylate of MoaD, thus forming thiocarboxylated MoaD that is able to assemble into an active MPT synthase complex [50]. Among three cysteine desulfurases tested, CsdB/SufS revealed the highest activity in MPT synthase activation, but up to now the in vivo sulfur source of bacterial MPT dithiolate remains unknown. A redundant function of different persulfide-generating systems is possible [50].

The eukaryotic homologues of MoeB are MOCS3 in humans [93], Cnx5 in plants [94] and CnxF in *Aspergillus* [38]. In addition to a conserved MoeB domain, all these proteins are characterized by large C-terminal extensions forming a rhodanese-like domain (RLD), pointing to a different sulfur transfer mechanism between eukaryotes and bacteria (fig. 5). Rhodaneses (thiosulfate:cyanide sulfurtransferases) are widespread enzymes that catalyze in vitro the transfer of a sulfane sulfur atom from thiosulfate to cyanide. The biological role of rhodaneses is yet unknown, because their in vivo substrates have not been identified. In the active form, the sulfur is bound to a conserved cysteine residue as persulfide, and this cysteine is also conserved in the RLD of CnxF [38], Cnx5 [94] and MOCS3 [93].

Studies with human RLD of MOCS3 demonstrated its ability to catalyze the transfer of sulfur from thiosulfate to cyanide [93]. In a defined in vitro assay generating MPT from cPMP, the sulfurated form of MOCS3-RLD was able to provide the sulfur for the thiocarboxylation of MOCS2A, the small subunit of human MPT synthase [93] (fig. 5). Mutation of the putative persulfide-forming active-site cysteine residue Cys412 abolished the sulfurtransferase activity of MOCS3-RLD completely, showing the importance of this cysteine residue for catalysis. Recently, Matthies et al. [95] were able to demonstrate the presence of a persulfide bound to the catalytically active Cys412. Therefore, MOCS3 and its eukaryotic homologues can be seen as multi-functional and multi-domain proteins combining the adenylation of the small subunit (MoeB domain) with the subsequent sulfur transfer reaction (RLD).

Steps 3 and 4: Mo insertion

After synthesis of MPT, the chemical backbone is ready to bind and coordinate the Mo atom. Mo has to be taken up into the cell as molybdate for which high-affinity transporters have been described in bacteria [96]. Once inside the cell, a key question of Moco biosynthesis was whether it is molybdate that serves as donor for insertion of Mo into MPT or whether molybdate has to undergo intracellular processing prior to insertion.

One of the first observations of Moco mutants was the identification of molybdate-repairable phenotypes, pointing to a defect in molybdate uptake or Mo insertion [97]. *E. coli* mutants with complete suppression of phenotype were found to have a defect in the *mod* locus encoding a high-affinity uptake system. The ABC-type molybdate transporter is composed of ModA (24–25 kDa), a periplasmic monomeric oxyanion-binding protein, ModB (25 kDa), a dimeric integral transmembrane protein forming the anion chanel and ModC (38–39 kDa), a dimeric ATP-binding peripheral membrane-binding protein. Additional proteins encoded by the *mod* operon have functions in intracellular molybdate binding and regulation of gene expression. For example, *E. coli* ModE was found to control gene expression of the *mod* [98] as well as *moa* operon [99]. In contrast to the well-studied molybdate transport and homeostasis mechanism in bacteria, eukaryotic molybdate transport is still poorly understood. Only recently, in *Chlamydomonas rheinhardtii,* genetic evidence has suggested that a distinct high-affinity/low-capacity and low-affinity/high-capacity uptake system exists [100]. Based on the low-affinity co-transport of molybdate by sulfate and phosphate transporters in prokaryotes, one can assume that a member of this protein family might also act on molybdate in eukaryotes.

Molybdate-repairable mutants with partial restoration of Mo enzyme activity are impaired in Mo insertion. The *E. coli mog* locus encodes for MogA, for which a function in Mo insertion was proposed. In contrast, plant, fungal and mammalian molybdate-repairable mutants showed a mutation in one of the two conserved domains of proteins such as Cnx1 [101], CnxE [40] or gephyrin [43], respectively. All these proteins contain one MogA-homologous G domain and in addition a MoeA-homologous E domain. Based on these findings, *E. coli* MoeA was also proposed to participate in Mo insertion. The modular nature of these proteins pointed to functional 'cooperation' between the domains, such as substrate-product channeling. Based on recent biochemical and structural studies with plant Cnx1 [51, 102], substantial progress in the understanding of the metal transfer mechanism has been made (fig. 6). A novel biosynthetic intermediate, adenlyated MPT (MPT-AMP), has been identified, and consequently Mo insertion is divided into two separate reactions (steps 3 and 4).

Step 3: adenlyation of MPT

Cnx1 from *Arabidopsis thaliana* was identified by functional complementation of *E. coli mogA* mutants. Mutants in *mogA* were found to accumulate MPT [103], and consequently binding of the hypothetical substrate MPT to the MogA-homologous Cnx1 G domain (Cnx1G) was demonstrated [104]. Subsequent studies using Cnx1G

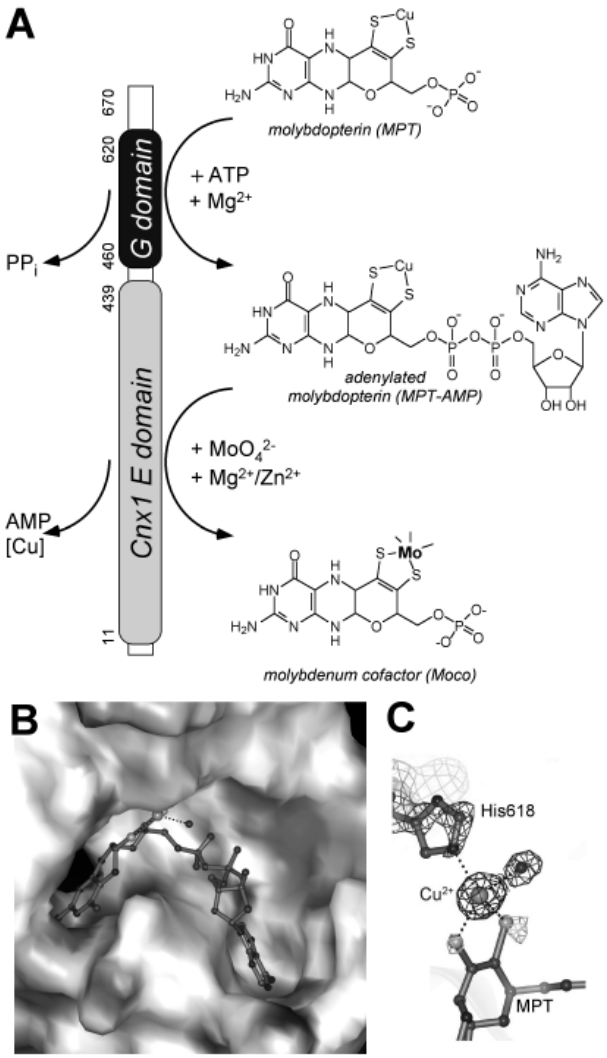

Figure 6. Adenylation of MPT and metal insertion. (A) Cnx1-catalyzed Mo insertion involves the synthesis of adenylated MPT, which is catalyzed by the C-terminal G domain using Mg-ATP. The product of Cnx1G, MPT-AMP, is transferred to Cnx1E, where MPT-AMP is hydrolyzed in a molybdate-dependent manner, thus yielding Moco. Net products of the Cnx1 reaction are pyrophosphate, AMP and copper. (B) Molecular surface of Cnx1G S583A variant with bound MPT-AMP. Electrostatic surface potential with +/−11 k_BT as borders for electropositive (blue) and negative (red) regions. (C) Anomalous Fourier maps (colored) of Cnx1G S583A variant surrounding the Cu site. Dashed lines indicate bonds between Cu and coordinating atoms. Anomalous Fourier maps are derived from diffraction data recorded at 1.05 Å (blue, 5.0 sigma) and 1.75 Å (yellow, 3.0 sigma). Modified after Kuper et al. (2004) [51].

variants indicated functions in MPT binding and catalysis; the latter was believed to be associated with Mo insertion [105]. However, binding of molybdate could not be detected [101]. Crystal structures of MogA [61] and Cnx1G [106] have been determined, and functionally important residues have been mapped. A large surface depression within the globular α/β proteins indicated a clear separation between MPT binding and residues important for catalysis [107].

Crystal structures of Cnx1G wild-type and mutant variants in complex with MPT confirmed the proposed binding of MPT [51]. These structures were obtained from proteins that were purified from *E. coli* under conditions maintaining the binding of the highly oxygen-sensitive substrate MPT. The crystal structure of a variant (S583A) with a gain of function revealed a novel intermediate in Moco biosynthesis because an adenosine covalently bound via a pyrophosphate bond to the C4' carbon of MPT was found, thus forming adenylated MPT [51] (fig. 6B). Catalytically inactive variants of Cnx1G could not copurify MPT-AMP. Subsequently, the Mg^{2+}- and ATP-dependent in vitro synthesis of MPT-AMP was demonstrated [102] (fig. 6A). As usual for this kind of reaction, released pyrophosphate inhibited the adenylation reaction. In general, MPT adenylation by Cnx1G was relatively slow, with a k_{cat} of 0.001 s^{-1} and a K_m of 60 μM ATP. Based on the ability of Cnx1G to reconstitute *mogA* mutants, one can conclude that both proteins catalyze the conserved MPT adenylation, which is essential for and takes place prior to metal insertion.

An unexpected observation in the substrate- and product-bound Cnx1G structures was the identification of copper bound to the MPT dithiolate sulfurs [51], whose nature was confirmed by anomalous scattering of the metal (fig. 6C). In both structures the copper atom shows tetragonal coordination with two waters as additional ligands in the MPT-bound state, while one of these waters is replaced by a histidine in the MPT-AMP-bound structure. Up to now the function of this novel MPT ligand is unknown, but copper might play a role in sulfur transfer to cPMP, in protecting the MPT dithiolate from oxidation and/or presenting a suitable leaving group for Mo insertion. In vitro studies with Cnx1G-bound MPT-AMP revealed inhibition of Moco synthesis in the presence of excess copper, providing a metabolic link to copper homeostasis disorders [51].

Step 4: the metal insertion reaction

With the identification of MPT-AMP as a novel and mechanistically important intermediate of Moco biosynthesis, the question arose of the function of the other Cnx1E domain (Cnx1E) and the mechanism of conversion of MPT-AMP into Moco. In vivo studies based on transcriptional regulation of the *E. coli mod* operon already showed MPT-dependent binding of molybdate to Cnx1 [108]. In vitro experiments using MPT-AMP-loaded Cnx1G demonstrated Mg-dependent synthesis of Moco by using desalted *nit-1* protein extract to monitor Moco formation (*nit-1* assay). This activity was significantly enhanced by the addition of Cnx1E, suggesting

a function in Mo insertion [51]. Recently, we achieved a molybdate-dependent hydrolysis of MPT-AMP [52; unpublished observations]. Cleavage of AMP was coupled to the metal exchange reaction where copper was released and Mo was transferred to MPT, thus yielding Moco (fig. 6A). More experiments are needed to uncover the molecular mechanism of this novel nucleotide-assisted Mo insertion reaction.

For the Cnx1E-corresponding *moeA* mutant, suppression of Moco deficiency was found in low-sulfur medium [109]. Under those conditions genes encoding proteins of the sulfate assimilation pathway are induced [110]. Molybdate is known to act as a substrate analogue of ATP sulfurylase (molybdolysis [111]), the first enzyme in this pathway catalyzing the adenylation of sulfate [112]. Therefore, high expression of ATP sulfurylase could result in transient formation of adenylated molybdate, a species that is supposed to be highly reactive and might be related to the reaction of MoeA during Moco synthesis. Based on these findings and the known adenylation of MPT, a mechanism is proposed that involves an adenylated molybdate intermediate during metal insertion [52].

When comparing eukaryotic and prokaryotic Mo transfer to MPT, differences become obvious. All bacterial Mo enzymes that belong to the DMSO reductase family contain a bis-MPT-based cofactor that should require a different metal insertion process than a mono-MPT-based cofactor as found in all eukaryotes as well as bacterial enzymes of the XOR family (fig. 1). The majority of *E. coli* Mo enzymes contain a bis-MPT-type cofactor, and *moeA* mutants show no molybdate repairable phenotype with respect to the activity of those enzymes [109]. Recently, a novel MPT-type oxidoreductase was identified in *E. coli* that belongs to the eukaryotic sulfite oxidase family of Mo enzymes [113]. As in other bacteria, such as *Rhodobacter capsulatus moeA,* mutants show molybdate suppression, but only for enzymes of the XOR family [114]. One can argue that this phenotype is only observed for MPT-dependent Mo enzymes. Interestingly, the function of *E. coli* MoeA (vis à vis bis-MPT enzymes) cannot be reconstituted by Cnx1E [101], which is further support for functional diversity between bacterial and eukaryotic Mo insertion. In vitro studies using purified MoeA demonstrated the formation of eukaryotic Moco (mono-Moco) by using MPT and molybdate [115]. It remains open whether this activity is physiologically relevant, as MPT-AMP synthesis was bypassed in this study. The observed direct transfer of Mo to MPT might also be part of the unique function of MoeA to generate a bis-MPT-based cofactor. In summary, one can conclude that both domains (G and E) or proteins MogA and MoeA are essential to catalyze a two-step reaction leading to metal transfer to MPT in order to form Moco.

Step 5: maturation of Moco

Moco biosynthesis can be considered complete once the metal is incorporated into the pterin moiety [36]. However, additional modifications must occur before the cofactor is inserted into the appropriate apo-enzyme and/or Moco is able to promote catalysis. In brief, bacterial cofactors of the DMSO reductase family have a guanine attached, forming the bis-MGD-type cofactor [10], while carbon monoxide dehydrogenases are modified by cytosine, forming a mono-MCD cofactor [116]. In addition, the latter is unique in terms of its metal center because via a third sulfur ligand a second nuclear center, copper, bridges the Mo to a protein cysteine residue [11]. Enzymes of the XOR family are characterized by a unique third sulfur ligand, while in enzymes of the SO family the position of the XO sulfur is occupied by the sulfur of a conserved cysteine, which covalently links Mo to the apo-protein [117].

The *mob* locus is essential for the nucleotide attachment in *E. coli* Moco biosynthesis [118, 119]. MobA catalyzes the conversion of MPT and GTP to MGD [120], while MobB is a GTP-binding protein with weak GTPase activity [121]. Temple and Rajagopalan [53] have shown that in vitro only MobA, GTP, $MgCl_2$ and Mo-MPT are required for assembly of active DMSO reductase from apo-protein. Under those conditions, the formation of Mo-MGD can proceed in the absence of any acceptor apo-protein, and MobB seems not to be essential for MGD synthesis in vitro. However, in vivo other factors, such as chaperones like the NR-specific NarJ protein from *E. coli,* may play an important role in this step [122]. Interestingly, dinculeotide formation was found to be molybdate dependent, because depletion of molybdate from the culture medium or addition of tungstate abolished MCD synthesis in *Hydrogenophaga pseudoflava* [123]. Under those conditions the corresponding nucleotides were still bound to the apo-protein due to their high affinity as detected in DMSO reductase [124], NR [125] or CO dehydrogenase [123]. Now we know that the other nucleotide-substituted intermediate, MPT-AMP, is processed in a molybdate-dependent manner, which is a prerequisite for final maturation of the bacterial cofactor. The crystal structure of *E. coli* MobA [63, 126] shows an α/β architecture with a nuleotide-binding Rossman fold formed by the N-terminal half of the protein. Lake et al. [63] have crystallized MobA in an octameric stage, while the conformation in solution is monomeric, which was seen in another structure determined by Stevenson et al. [126]. The active site was defined by highly conserved residues as well as by co-crystallization of MobA with GTP, which is bound in the N-terminal part [63]. The binding site of MPT, formed by another set of highly conserved residues, was modeled according to the position of GTP and confirmed by structure-guided mutagenesis [54].

Enzymes of the XOR family require a third sulfur ligand that does not originate from the apo-protein. For rat and fly, the early work of Wahl et al. [127] demonstrated that in vitro this sulfur can be spontaneously lost or can be removed from AO and XDH by cyanide treatment, generating an inactive enzyme. The reaction, however, is reversible, and the enzyme can be reactivated by sulfide treatment under reducing conditions. In vivo, this terminal sulfur has to be added by a separate enzymatic reaction, catalyzed by a Moco sulfurase (MCSU). MCSU activities were described for *D. melanogaster* (Ma-l [127]), *A. nidulans* (HxB [128]), cattle (MCSU [55]), plants (ABA3 [129]) and humans (MCSU [130]. Loss of MCSU in animals, including humans, results in xanthinuria type II [130]. Only for the plant enzyme ABA3 are biochemical data available [56], providing evidence for Moco sulfurase-catalyzed activation of enzymes of the XOR family (fig. 7). ABA3 performed a sulfur transfer with a Moco-containing aldehyde oxidase; so at least under in vitro conditions, ABA3 is able to act directly on an enzyme-bound Moco. However, it remains unclear whether ABA3 sulfurates Moco in vivo before or after insertion into apo-enzymes. The N-terminus of ABA3 shares significant homologies to the bacterial cysteine sulfurases [56] SufS, IscS and NifS. In a pyridoxal phosphate-dependent mechanism of trans-sulfuration, an ABA3-bound persulfide, resulting from the desulfuration of free L-cysteine, is likely to be transferred to the Mo center [131] (fig. 7). The C-terminal domain of ABA3 is probably responsible for mediating the contact between XDH or AO and the trans-sulfurase domain of ABA3 [56, 132]. The terminal sulfuration step seems to be an interesting regulatory switch point, because the activity of ABA3 could control the amount of functional XDH/AO molecules in the cell that in turn would control cellular levels of e.g. hormones in the case of plant AO. In fact, transcription of the *aba3* gene was found to be inducible by drought and salt stress in plants [56] as well as by abscisic acid [133].

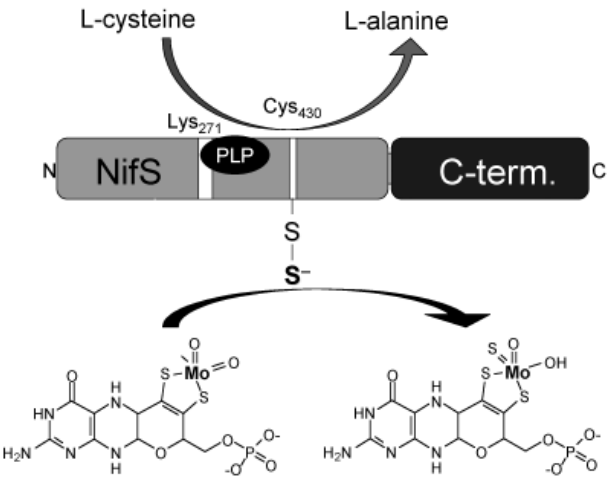

Figure 7. Moco sulfuration. Moco in enzymes of the XOR family is sulfurated before it is catalytically active. In eukaryotes Moco sulfurases such as ABA3 from *A. thaliana* catalyze this reaction. ABA3 contains an N-terminal NifS-like domain that binds pyridoxal phosphate (PLP) via a conserved lysine residue and desulfurates cysteine to alanine, thereby generating a cysteine persulfide. The latter is believed to be the source of sulfur, which is transferred to Moco. It is still unknown whether this sulfuration occurs before or after cofactor insertion into the apo-enzyme. The function of the C-terminal domain (C-term.) is unknown but might be involved in Moco transfer or apo-enzyme binding.

Storage and transfer of Moco

After synthesis and maturation Moco has to be incorporated into the appropriate apo-enzyme. Moco (or MGD/MCD) can either be transferred directly from the Moco-synthetic machinery or by a Moco carrier and/or storage protein. There are several indications that subsequent to Moco biosynthesis such proteins could take over the task to buffer supply and demand of Moco. Such proteins were described for *Rhodospirillum rubrum* [134], *E. coli* [135] and *C. reinhardtii* [60].

The initial evidence for a Moco-synthetic machinery came from studies with *E. coli*, where Magalon et al. [58] have used a two-hybrid approach to identify protein-protein interaction between proteins catalyzing the final steps of Moco biosynthesis. Multiple interactions between MogA, MoeA, MobA and MobB were found. Interactions between MoeA and MogA as well as MoeA and MobA were abolished in the absence of MPT, which is in line with recent findings regarding the function of Cnx1. MoeA should act on MPT-AMP, the reaction product of MogA, while MobA acts on Moco (bis-Moco), the product of MoeA. Binding between these proteins seems to be weak, as up to now no direct biochemical evidence has been presented confirming this cellular protein network. However, at least the interaction between MogA and MoeA might already point to their functional cooperation, a reason that was the driving force for fusing both proteins in a multi-domain protein as found in eukaryotes. Using the same method, further interactions between Moco-synthetic proteins and apo-NR were described that might point to a direct transfer of bis-MGD from the machinery to apo-enzymes [59]. In the case of bacterial NR, specific chaperones such as NarJ are essential for efficient Mo enzyme maturation [122]. Also in eukaryotes the existence of a multi-enzyme Moco-synthetic complex has been proposed, which might be anchored and compartmentalized via the cytoskeleton. The two-domain protein Cnx1 is believed to play a crucial role in the process [101].

Among eukaryotes, first in the green alga *C. rheinhardtii,* an activity of a Moco carrier protein (MCP) was described [136]. Later the activity was purified, and a

16-kDa protein was identified, which was able to bind and protect Moco against oxidation [60]. MCP activity was measured with the *nit-1* reconstitution assay without any denaturing procedure, indicating that bound Moco is delivered to apo-NR. It is unknown whether MCP is also able to donate Moco to Mo enzymes other than NR. *C. rheinhardtii* MCP forms a homotetramer in solution and is homologous to bacterial proteins with unknown function that contain a predicted nucleotide-binding Rossman fold [137]. In eukaryotes, proteins with low homologies are found that are classified as lysine decarboxylase-like proteins forming multi-gene families. In *A. thaliana*, for example, up to eight different highly conserved members were found.

Regarding Moco insertion into NR or SO, nothing is known about a specific mechanism leading to the covalent attachment of the metal to the enzyme-specific and conserved cysteine residue that forms the third sulfur ligand of Moco. In vitro NR, at least, as well as SO are able to bind Moco very efficiently, with reconstitution times of less than an hour [138]. Regarding the mechanism of Moco ligation to NR and SO, it is also important to state that the oxygen coordination of Moco as generated by the action of Cnx1E is unknown. It is possible that due to the proposed nucleotide-assisted metal transfer mechanism three oxygen atoms remain bound in mature Moco, where one of them is replaced by the terminal sulfur ligand in XOR-like enzymes, while in SO-like enzymes this position is occupied by a cysteine.

Human Moco deficiency

Molybdenum cofactor deficiency in humans results in the simultaneous loss of SO, XOR and AO activity. The severe phenotype of Moco-deficient patients is characterized by progressive neurological damage leading in most cases to early childhood death, mainly caused by the deficiency of SO that protects the organism, in particular the brain, from elevated levels of toxic sulfite [139]. All forms of Moco deficiency are inherited as autosomal recessive traits and occur in all racial groups. So far, disease-causing mutations have been identified in three of the four known Moco-synthetic human genes: *MOCS1*, *MOCS2* and *GEPH* [140]. The remarkable bicistronic architecture of *MOCS1* and *MOCS2* has already been discussed. The protein gephyrin, encoded by a third locus, is highly homologous to the plant protein Cnx1 and was originally identified in a completely different context: gephyrin is tightly associated with inhibitory neuroreceptors in the post-synaptic membrane [141] and plays a crucial role in the organization of postsynaptic membrane specializations [142]. However, several studies have unequivocally demonstrated that gephyrin is also a multifunctional protein catalyzing Moco biosynthesis, in addition to its brain-specific function. Gephyrin is able to reconstitute bacterial, mammalian and plant Moco biosynthesis [43], and loss of gephyrin causes Moco deficiency in mice [143] and humans [144].

The first case of a combined deficiency of SO and XDH was described by Duran et al. [145]. To this end, more than 100 cases have been diagnosed, and approximately 50 unrelated families have been analyzed at the molecular level [140]. A total of 32 different disease-causing mutations, including several common to more than one family, have been identified in Moco-deficient patients and their relatives. The incidence, however, is estimated to be higher than these numbers would indicate, as it is likely that many cases remain unrecognized. Affected individuals normally come to clinical attention shortly after birth because of a failure to thrive, and seizures that are often unresponsive to treatment. Elevated urinary sulfite can be detected using dipsticks; however, a more reliable diagnostic test measures urinary S-sulfocysteine by mass spectrometry [146]. Later, if they survive the neonatal period, progressive loss of white matter in the brain, feeding difficulties and dislocated ocular lenses are observed [139]. The clinical symptoms may result from sulfite toxicity, tissue-specific sulfate deficiency or a combination of both. Although milder cases have been reported, most patients die in early childhood, and some survive only a few days because currently no effective therapy is available.

The biochemical analysis of cultured fibroblasts from different patients led to the description of two complementation groups [147]. Co-cultivation experiments indicated that cells from group B patients excreted a relatively stable and diffusible precursor (later identified as precursor Z/cPMP) that could be converted to active Moco by cells from group A patients. These experiments already suggested that intermediates of the Moco biosynthetic pathway are able to cross the plasma membrane. Only recently a third type of human mutation was identified that accumulates MPT and thus is defective in the insertion of Mo into MPT [144]. With the knowledge of the genes essential for human Moco biosynthesis, the identification of mutations became possible. Moreover, mutations in the *MOCS1* gene were already identified using prenatal diagnosis [148] in cases with a family history in Moco deficiency.

Therapy of Moco deficiency

In order to study human Moco deficiency in greater detail, an animal model was generated with a defect in the first step of the biosynthesis resembling the most frequent type of mutation classified as group A patients [149]. Among all known Moco-deficient patients studied so far, most patients harbor mutations in the *MOCS1*

gene [140], whose murine homologue was disrupted by homologous recombination with a targeting vector. Similar to humans, heterozygous mice displayed no symptoms, but homozygous animals died between days 1 and 11 after birth [149]. Due to the mutation in the first step of the pathway, no MPT or active Moco was detectable, and consequently all Mo enzyme activities were absent. Organ abnormalities were not observed. In summary, *MOCS1*-deficient mice present a suitable model for genetic and/or biochemical therapy approaches.

In contrast to other disorders of pterin metabolism, such as the deficiency of tetrahydrobiopterin [150], the essential cofactor of aromatic amino acid hydroxylases and NO synthases, Moco deficiency cannot be treated by supplementation with the cofactor. Moco is extremely unstable outside the protecting environment of an apo-Mo enzyme. Its half-life is only a few minutes in aqueous solutions with neutral pH [151]. In addition, to this end no chemical synthesis of Moco or any of its intermediates has been successful so far, which hampers its large-scale production for therapeutic use.

Among all intermediates of the Moco synthetic pathway, cPMP has the highest stability at neutral pH values ($t_{1/2}$ = 1 h) due to its unique pyranopterin nature and the absence of the very reactive dithiolate sulfurs and Mo. Therefore, a method was established to produce and purify relatively large amounts of cPMP in *E. coli* in order to use cPMP for a substitution therapy of Moco deficiency [152]. Repeated injections of cPMP into *MOCS1*-deficient mice resulted in a dose-dependent extension of life span [152]. MPT levels and Mo enzyme activities were partially restored. Stopping cPMP treatment at any time resulted in a progressive reduction of MPT levels and Mo enzyme activities and death of the animal 10–15 days after receiving the last injection. As two-thirds of the known Moco-deficient patients belong to deficiency group A [75, 140] and all components necessary for cPMP conversion into MPT are present in those patients [147, 153], purified cPMP represents the first potentially effective drug for the majority of Moco patients. Since the *MOCS1*-deficient mice match the phenotype of human patients with respect to biochemical parameters, the correction of the human deficiency by cPMP delivery appears promising at the level of enzymatic restoration and metabolic correction. It remains to be seen whether delayed onset of the described therapy will still allow reversal of neurological damage. The cPMP therapy also provides the basis to study the progression or reversal of this damage in detail, as Moco deficiency can be induced in the animal model at any time by altering the dose of the drug. Also MPT or MPT-AMP are suitable candidates for drug development that might be delivered directly or in a protein-bound state in order to treat patients of group B.

Future directions and open questions

In the past 10 years molecular biology, biochemistry, structural biology, chemistry as well as cell biology have brought great progress in understanding Moco function and biosynthesis. Regardless of many breaking results, there is still a large number of unresolved questions that need to be answered in the future in order to understand the complex biosynthetic pathway of Moco. While the synthesis of pteridines and folates is well known, the chemistry underlying cPMP formation is still enigmatic, and proposed theories have to be proven. The role of SAM in catalysis as well as the function of the other MoaC-like proteins is of great interest. Also the functional importance of the complex splicing of the human *MOCS1* gene is essential to uncover. What are the reaction intermediates of cPMP synthesis? And, given that cPMP is of great medical relevance for the treatment of Moco-deficient patients, can it be synthesized chemically? What can MPT synthesis mechanism tell us about the state of copper bound to MPT, and what is the role of copper in Moco synthesis in general? Is there any metabolic link between Mo and copper metabolism? The source of sulfur for the MPT dithiolate should be identified. Furthermore, the detailed mechanism of Mo insertion has to be resolved. Is adenlyated molybdate the reaction intermediate of the metal insertion reaction or is molybdate activated in a different way? How does Mo attach to two MPTs in order to get bis-Moco? What is the structure of Moco outside a Mo enzyme, and how is Moco inserted in apo-Mo enzymes? Is there a general mechanism or does it depend on the modifications? At which cellular compartment is Moco synthesized, and how is Moco biosynthesis regulated? Finally, an issue related to Moco synthesis is the formation of the structurally similar tungsten cofactor mainly found in Archaea. Here the main question regards the mechanisms for a metal-selective Mo or W insertion during Moco or Wco synthesis.

Acknowledgement. I thank Katrin Fischer for help preparing figures and reading the manuscript and Ralf R. Mendel, Florian Bittner, José Santamaria and Jan Winking for critical reading and helpful discussions. I am deeply grateful to all my co-workers past and present. My own work was supported by the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie and the International Graduate Program of the Technical University of Braunschweig.

1 Rees D. C. (2002) Great metalloclusters in enzymology. Annu. Rev. Biochem. **71:** 221–246
2 Rajagopalan K. V. and Johnson J. L. (1992) The pterin molybdenum cofactors. J. Biol. Chem. **267:** 10199–10202
3 Allen R. M., Chatterjee R., Madden M. S., Ludden P. W. and Shah V. K. (1994) Biosynthesis of the iron-molybdenum cofactor of nitrogenase. Crit. Rev. Biotechnol. **14:** 225–249
4 Hille R. (2002) Molybdenum and tungsten in biology. Trends Biochem. Sci. **27:** 360–367
5 Hille R. (1996) The mononuclear molybdenum enzymes. Chem. Rev. **96:** 2757–2816

6 Kisker C., Schindelin H., Pacheco A., Wehbi W. A., Garrett R. M., Rajagopalan K. V. et al. (1997) Molecular basis of sulfite oxidase deficiency from the structure of sulfite oxidase. Cell **91:** 973–983

7 Campbell W. H. (2001) Structure and function of eukaryotic NAD(P)H:nitrate reductase. Cell. Mol. Life Sci. **58:** 194–204

8 Garattini E., Mendel R., Romao M. J., Wright R. and Terao M. (2003) Mammalian molybdo-flavoenzymes, an expanding family of proteins: structure, genetics, regulation, function and pathophysiology. Biochem J. **372:** 15–32

9 Seo M., Peeters A. J., Koiwai H., Oritani T., Marion-Poll A., Zeevaart J. A. et al. (2000) The arabidopsis aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc. Nat. Acad. Sci. USA **97:** 12908–12913

10 Moura J. J., Brondino C. D., Trincao J. and Romao M. J. (2004) Mo and W bis-MGD enzymes: nitrate reductases and formate dehydrogenases. J. Biol. Inorg. Chem. **9:** 791–799

11 Dobbek H., Gremer L., Kiefersauer R., Huber R. and Meyer O. (2002) Catalysis at a dinuclear [CuSMo(==O)OH] cluster in a CO dehydrogenase resolved at 1.1-A resolution. Proc. Natl. Acad. Sci. USA **99:** 15971–15976

12 Pateman J. A., Cove D. J., Rever B. M. and Roberts D. B. (1964) A common cofactor for nitrate reductase and xanthine dehydrogenase wich also regulates the synthesis of nitrate reductase. Nature **201:** 58–60

13 Cove D. J. and Pateman J. A. (1963) Independently segregating genetic loci concerned with nitrate reductase activity in *Aspergillus nidulans*. Nature **198:** 262–263

14 Tomsett A. B. and Garrett R. H. (1980) The isolation and characterization of mutants defective in nitrate assimilation in Neurospora crassa. Genetics **95:** 649–660

15 Glaser J. H. and DeMoss J. A. (1971) Phenotypic restoration by molybdate of nitrate reductase activity in chlD mutants of Escherichia coli. J. Bacteriol. **108:** 854–860

16 Warner C. K. and Finnerty V. (1981) Molybdenum hydroxylases in *Drosophila*. II Molybdenum cofactor in xanthine dehydrogenase, aldehyde oxidase and pyridoxal oxidase. Mol. Gen. Genet **184**

17 Müller A. J. and Mendel R. R. (1989) Biochemical and somatic cell genetics of nitrate reductase in *Nicotiana*. In: Molecular and Genetic Aspects of Nitrate Assimilation, pp. 166–185, Wray J. L. and Kinghorn J.R. (eds.), Oxford University Press, Oxford

18 Johnson J. L., Waud W. R., Rajagopalan K. V., Duran M., Beemer F. A. and Wadman S. K. (1980) Inborn errors of molybdenum metabolism: combined deficiencies of sulfite oxidase and xanthine dehydrogenase in a patient lacking the molybdenum cofactor. Proc. Natl. Acad. Sci. USA **77:** 3715–3719

19 Gabard J., Pelsy F., Marion-Poll A., Caboche M., Saalbach I., Grafe R. et al. (1988) Genetic analysis of nitrate reductase deficient mutants of Nicotiana plumbaginifolia: Evidence for six complementation groups among 70 classified molybdenum cofactor deficient mutants. Mol. Gen. Genet. **213:** 275–281

20 Sorger G. J., Giles N. H. (1965) Genetic control of nitrate reductase in *Neurospora crassa*. Genetics **52:** 777–788

21 Ketchum P. A., Cambier H. Y., Frazier W. A., 3rd, Madansky C. H., Nason A. (1970) In vitro assembly of Neurospora assimilatory nitrate reductase from protein subunits of a Neurospora mutant and the xanthine oxidizing or aldehyde oxidase systems of higher animals. Proc. Natl. Acad. Sci. USA **66:** 1016–1023

22 Nason A., Antoine A. D., Ketchum P. A., Frazier W. A., 3rd, Lee D. K. (1970) Formation of assimilatory nitrate reductase by in vitro inter-cistronic complementation in Neurospora crassa. Proc. Natl. Acad. Sci. USA **65:** 137–144

23 Nason A., Lee K. Y., Pan S. S., Ketchum P. A., Lamberti A., DeVries J. (1971) Invitro formation of assimilatory reduced nicotinamide adenine dinucleotide phosphate: nitrate reductase from a Neurospora mutant and a component of molybdenum-enzymes. Proc. Natl. Acad. Sci. USA **68:** 3242–3246

24 Pienkos P. T., Shah V. K., Brill W. J. (1977) Molybdenum cofactors from molybdoenzymes and in vitro reconstitution of nitrogenase and nitrate reductase. Proc. Nat. Acad. Sci. USA **74:** 5468–5471

25 Mendel R. R., Alikulov Z. A., Lvov N. P., Müller A. J. (1981) Presence of the molybdenum-cofactor in nitrate reductase-deficient mutant cell lines of Nicotiana tabacum. Mol. Gen. Genet. **181:** 395–399

26 Mendel R. R., Buchanan R. J., Wray J. L. (1984) Characterization of a new type of molybdenum cofactor-mutant in cell cultures of *Nicotiana tabacum*. Mol. Gen. Genet. **195:** 186–189

27 Wahl R. C., Hageman R. V., Rajagopalan K. V. (1984) The relationship of Mo, molybdopterin, and the cyanolyzable sulfur in the Mo cofactor. Arch. Biochem. Biophys. **230:** 264–273

28 Alikulov Z. A., Mendel R. R. (1983) Molybdenum cofactor from tobacco cell cultures and milk xanthine oxidase: Involvement of sulfhydryl groups in dimerization activity of cofactor. Biochem. Physiol. Pflanzen. **179:** 693–705

29 Mendel R. R. (1983) Release of molybdenum co-factor from nitrate reductase and xanthine oxidase by heat treatment. Phytochemistry **22:** 817–819

30 Johnson J. L., Hainline B. E., Rajagopalan K. V., Arison B. H. (1984) The pterin component of the molybdenum cofactor. Structural characterization of two fluorescent derivatives. J. Biol. Chem. **259:** 5414–5422

31 Johnson J. L., Rajagopalan K. V. (1982) Structural and metabolic relationship between the molybdenum cofactor and urothione. Proc. Natl. Acad. Sci. USA **79:** 6856–6860

32 Kramer S. P., Johnson J. L., Ribeiro A. A., Millington D. S., Rajagopalan K. V. (1987) The structure of the molybdenum cofactor. Characterization of di- (carboxamidomethyl)molybdopterin from sulfite oxidase and xanthine oxidase. J. Biol. Chem. **262:** 16357–16363

33 Nieter Burgmayer S. J., Pearsall D. L., Blaney S. M., Moore E. M., Sauk-Schubert C. (2004) Redox reactions of the pyranopterin system of the molybdenum cofactor. J Biol Inorg Chem **9:** 59–66

34 Mendel R. R. (1992) The plant molybdenum cofactor (MoCo) – its biochemical and molecular genetics. In: Plant Biotechnology and Development – Current Topics in Plant Molecular Biology, vol. 1, pp. 11–16, Gresshoff P. M. (ed.), CRC Press, Boca Raton

35 Shanmugam K. T., Stewart V., Gunsalus R. P., Boxer D. H., Cole J. A., Chippaux M. et al. (1992) Proposed nomenclature for the genes involved in molybdenum metabolism in Escherichia coli and Salmonella typhimurium [letter]. Mol. Microbiol. **6:** 3452–3454

36 Mendel R. R., Schwarz G. (2002) Biosynthesis and molecular biology of the molybdenum cofactor (Moco). Met. Ions Biol. Syst. **39:** 317–368

37 Unkles S. E., Smith J., Kanan G. J., Millar L. J., Heck I. S., Boxer D. H. et al. (1997) The Aspergillus nidulans cnxABC locus is a single gene encoding two catalytic domains required for synthesis of precursor Z, an intermediate in molybdenum cofactor biosynthesis. J. Biol. Chem. **272:** 28381–28390

38 Appleyard M. V., Sloan J., Kana'n G. J., Heck I. S., Kinghorn J. R., Unkles S. E. (1998) The Aspergillus nidulans cnxF gene and its involvement in molybdopterin biosynthesis. Molecular characterization and analysis of in vivo generated mutants. J. Biol. Chem. **273:** 14869–14876

39 Unkles S. E., Heck I. S., Appleyard M. V., Kinghorn J. R. (1999) Eukaryotic molybdopterin synthase. Biochemical and molecular studies of Aspergillus nidulans cnxG and cnxH mutants. J. Biol. Chem. **274:** 19286–19293

40 Millar L. J., Heck I. S., Sloan J., Kana'n G. J., Kinghorn J. R., Unkles S. E. (2001) Deletion of the cnxE gene encoding the gephyrin-like protein involved in the final stages of molybdenum cofactor biosynthesis in Aspergillus nidulans. Mol. Genet. Genomics **266:** 445–453

41 Reiss J., Cohen N., Dorche C., Mandel H., Mendel R. R., Stallmeyer B. et al. (1998) Mutations in a polycistronic nuclear gene associated with molybdenum cofactor deficiency. Nat. Genet. **20:** 51–53

42 Stallmeyer B., Drugeon G., Reiss J., Haenni A. L., Mendel R. R. (1999) Human molybdopterin synthase gene: identification of a bicistronic transcript with overlapping reading frames. Am. J. Hum. Genet. **64:** 698–705

43 Stallmeyer B., Schwarz G., Schulze J., Nerlich A., Reiss J., Kirsch J. et al. (1999) The neurotransmitter receptor-anchoring protein gephyrin reconstitutes molybdenum cofactor biosynthesis in bacteria, plants, and mammalian cells. Proc. Natl. Acad. Sci. USA **96:** 1333–1338

44 Hanzelmann P., Schindelin H. (2004) Crystal structure of the S-adenosylmethionine-dependent enzyme MoaA and its implications for molybdenum cofactor deficiency in humans. Proc. Natl Acad. Sci. USA **101:** 12870–12875

45 Haenzelmann P., Hernandez H. L., Menzel C., Garcia-Serres R., Huynh B. H., Johnson M. K. et al. (2004) Characterization of MOCS1A, an oxygen-sensitive iron-sulfur protein involved in human molybdenum cofactor biosynthesis. J. Biol. Chem. **279**: 34721–34732

46 Rudolph M. J., Wuebbens M. M., Rajagopalan K. V. and Schindelin H. (2001) Crystal structure of molybdopterin synthase and its evolutionary relationship to ubiquitin activation. Nat. Struct. Biol. **8:** 42–46

47 Gutzke G., Fischer B., Mendel R. R. and Schwarz G. (2001) Thiocarboxylation of molybdopterin synthase provides evidence for the mechanism of dithiolene formation in metal-binding pterins. J. Biol. Chem. **276:** 36268–36274

48 Lake M. W., Wuebbens M. M., Rajagopalan K. V. and Schindelin H. (2001) Mechanism of ubiquitin activation revealed by the structure of a bacterial MoeB-MoaD complex. Nature **414:** 325–329

49 Leimkühler S., Wuebbens M. M. and Rajagopalan K. V. (2001) Characterization of Escherichia coli MoeB and its involvement in the activation of molybdopterin synthase for the biosynthesis of the molybdenum cofactor. J. Biol. Chem. **276:** 34695–34701

50 Leimkuhler S. and Rajagopalan K. V. (2001) A sulfurtransferase is required in the transfer of cysteine sulfur in the in vitro synthesis of molybdopterin from precursor Z in Escherichia coli. J. Biol. Chem. **276:** 22024–22031

51 Kuper J., Llamas A., Hecht H. J., Mendel R. R. and Schwarz G. (2004) Structure of molybdopterin-bound Cnx1G domain links molybdenum and copper metabolism. Nature **430:** 806–806

52 Reference removed in proof

53 Temple C. A. and Rajagopalan K. V. (2000) Mechanism of assembly of the Bis(Molybdopterin guanine dinucleotide)molybdenum cofactor in Rhodobacter sphaeroides dimethyl sulfoxide reductase. J. Biol. Chem. **275:** 40202–40210

54 Guse A., Stevenson C. E., Kuper J., Buchanan G., Schwarz G., Giordano G. et al. (2003) Biochemical and structural analysis of the molybdenum cofactor biosynthesis protein MobA. J. Biol. Chem. **278:** 25302–25307

55 Watanabe T., Ihara N., Itoh T., Fujita T. and Sugimoto Y. (2000) Deletion mutation in *Drosophila ma-l* homologous, putative molybdopterin cofactor sulfurase gene is associated with bovine xanthinuria type II. J. Biol. Chem. **275:** 21789–21792

56 Bittner F., Oreb M. and Mendel R. R. (2001) ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in Arabidopsis thaliana. J. Biol. Chem. **276:** 40381–40384

57 Fischer K., Barbier G. G., Hecht H.-J., Mendel R. R., Campbell W. H. and Schwarz G. (2005) Crystal structure of the yeast nitrate reductase molybdenum domain provides insight into eukaryotic nitrate assimilation. Plant Cell **17:** 1167–1179

58 Magalon A., Frixon C., Pommier J., Giordano G. and Blasco F. (2002) In vivo interactions between gene products involved in the final stages of molybdenum cofactor biosynthesis in Escherichia coli. J. Biol. Chem. **277:** 48199–48204

59 Vergnes A., Gouffi-Belhabich K., Blasco F., Giordano G. and Magalon A. (2004) Involvement of the molybdenum cofactor biosynthetic machinery in the maturation of the Escherichia coli nitrate reductase A. J Biol Chem **279:** 41398–41403

60 Witte C. P., Igeno M. I., Mendel R., Schwarz G. and Fernandez E. (1998) The Chlamydomonas reinhardtii MoCo carrier protein is multimeric and stabilizes molybdopterin cofactor in a molybdate charged form. FEBS Lett. **431:** 205–209

61 Liu M. T., Wuebbens M. M., Rajagopalan K. V. and Schindelin H. (2000) Crystal structure of the gephyrin-related molybdenum cofactor biosynthesis protein MogA from Escherichia coli. J. Biol. Chem. **275:** 1814–1822

62 Wuebbens M. M., Liu M. T. W., Rajagopalan K. V. and Schindelin H. (2000) Insights into molybdenum cofactor deficiency provided by the crystal structure of the molybdenum cofactor biosynthesis protein MoaC. Structure **8:** 709–718

63 Lake M. W., Temple C. A., Rajagopalan K. V. and Schindelin H. (2000) The crystal structure of the Escherichia coli MobA protein provides insight into molybdopterin guanine dinucleotide biosynthesis. J. Biol. Chem. **275:** 40211–40217

64 Xiang S., Nichols J., Rajagopalan K. V. and Schindelin H. (2001) The crystal structure of *Escherichia coli* MoeA and its relationship to the multifunctional protein gephyrin. Structure **9:** 299–310

65 Thony B., Auerbach G. and Blau N. (2000) Tetrahydrobiopterin biosynthesis, regeneration and functions. Biochem. J. **347 Pt 1:** 1–16

66 Bacher A., Eberhardt S., Eisenreich W., Fischer M., Herz S., Illarionov B. et al. (2001) Biosynthesis of riboflavin. Vitam. Horm. **61:** 1–49

67 Wuebbens M. M. and Rajagopalan K. V. (1995) Investigation of the early steps of molybdopterin biosynthesis in Escherichia coli through the use of in vivo labeling studies. J. Biol. Chem. **270:** 1082–1087

68 Rieder C., Eisenreich W., O'Brien J., Richter G., Götze E., Boyle P. et al. (1998) Rearrangement reactions in the biosynthesis of molybdopterin – An NMR study with multiply $^{13}C/^{15}N$ labelled precursors. Eur. J. Biochem. **255:** 24–36

69 Menendez C., Siebert D. and Brandsch R. (1996) MoaA of Arthrobacter nicotinovorans pAO1 involved in Mo-pterin cofactor synthesis is an Fe-S protein. FEBS Lett. **391:** 101–103

70 Rivers S. L., McNairn E., Blasco F., Giordano G. and Boxer D. H. (1993) Molecular genetic analysis of the moa operon of Escherichia coli K-12 required for molybdenum cofactor biosynthesis. Mol. Microbiol. **8:** 1071–1081

71 Sofia H. J., Chen G., Hetzler B. G., Reyes-Spindola J. F. and Miller N. E. (2001) Radical SAM, a novel protein superfamily linking unresolved steps in familiar biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. Nucleic Acids Res. **29:** 1097–1106

72 Hänzelmann P., Schwarz G. and Mendel R. R. (2002) Functionality of alternative splice forms of the first enzymes involved in human molybdenum cofactor biosynthesis. J. Biol. Chem. **277:** 18303–18312

73 Gray T. A. and Nicholls R. D. (2000) Diverse splicing mechanisms fuse the evolutionarily conserved bicistronic MOCS1A and MOCS1B open reading frames. RNA **6:** 928–936

74 Hoff T., Schnorr K. M., Meyer C. and Caboche M. (1995) Isolation of two Arabidopsis cDNAs involved in early steps of molybdenum cofactor biosynthesis by functional complementation of Escherichia coli mutants. J. Biol. Chem. **270:** 6100–6107

75 Reiss J. (2000) Genetics of molybdenum cofactor deficiency. Hum. Genet. **106:** 157–163

76 Wuebbens M. M. and Rajagopalan K. V. (1993) Structural characterization of a molybdopterin precursor. J. Biol. Chem. **268:** 13493–13498

77 Johnson J. L., Wuebbens M. M. and Rajagopalan K. V. (1989) The structure of a molybdopterin precursor. Characterization of a stable, oxidized derivative. J. Biol. Chem. **264:** 13440–13447
78 Doyle W. A., Burke J. F., Chovnick A., Dutton F. L., Whittle J. R. and Bray R. C. (1996) Properties of xanthine dehydrogenase variants from rosy mutant strains of Drosophila melanogaster and their relevance to the enzyme's structure and mechanism. Eur. J. Biochem. **239:** 782–795
79 Bertero M. G., Rothery R. A., Palak M., Hou C., Lim D., Blasco F. et al. (2003) Insights into the respiratory electron transfer pathway from the structure of nitrate reductase A. Nat. Struct. Biol. **10:** 681–687
80 Pfleiderer W. (1984) Chemistry of naturally occurring pterins. In: Folates and Pterins, vol. 2, pp. 43–114, Blakley R. L. and Benkovic S. J. (eds.), John Wiley, New York
81 Kaufman S. (1967) Metabolism of the phenylalanine hydroxylation cofactor. J. Biol. Chem. **242:** 3934–3943
82 Nieter Burgmayer S. J., Pearsall D. L., Blaney S. M., Moore E. M. and Sauk-Schubert C. (2004) Redox reactions of the pyranopterin system of the molybdenum cofactor. J. Biol. Inorg. Chem. **9:** 59–66
83 Santamaria-Araujo J. A., Fischer B., Otte T., Nimtz M., Mendel R. R., Wray V. et al. (2004) The tetrahydropyranopterin structure of the sulfur-free and metal-free molybdenum cofactor precursor. J. Biol. Chem. **279:** 15994–15999
84 Johnson M. E. and Rajagopalan K. V. (1987) In vitro system for molybdopterin biosynthesis. J Bacteriol **169:** 110–116
85 Pitterle D. M. and Rajagopalan K. V. (1993) The biosynthesis of molybdopterin in Escherichia coli. Purification and characterization of the converting factor. J. Biol. Chem. **268:** 13499–13505
86 Pitterle D. M., Johnson J. L. and Rajagopalan K. V. (1993) In vitro synthesis of molybdopterin from precursor Z using purified converting factor. Role of protein-bound sulfur in formation of the dithiolene. J. Biol. Chem. **268:** 13506–13509
87 Hershko A. and Ciechanover A. (1998) The ubiquitin system. Annu. Rev. Biochem. **67:** 425–479
88 Wuebbens M. M. and Rajagopalan K. V. (2003) Mechanistic and mutational studies of Escherichia coli molybdopterin synthase clarify the final step of molybdopterin biosynthesis. J. Biol. Chem. **278:** 14523–14532
89 Leimkuhler S., Freuer A., Araujo J. A., Rajagopalan K. V. and Mendel R. R. (2003) Mechanistic studies of human molybdopterin synthase reaction and characterization of mutants identified in group B patients of molybdenum cofactor deficiency. J. Biol. Chem. **278:** 26127–26134
90 McGrath J. P., Jentsch S. and Varshavsky A. (1991) *UBA1*: an essential yeast gene encoding ubiquitin-activating enzyme. EMBO J. **10:** 227–236
91 Hershko A. and Ciechanover A. (1992) The ubiquitin system for protein degradation. Annu. Rev. Biochem. **61:** 761–807
92 Begley T. P., Xi J., Kinsland C., Taylor S. and McLafferty F. (1999) The enzymology of sulfur activation during thiamin and biotin biosynthesis. Curr. Opin. Chem. Biol. **3:** 623–629
93 Matthies A., Rajagopalan K. V., Mendel R. R. and Leimkuhler S. (2004) Evidence for the physiological role of a rhodanese-like protein for the biosynthesis of the molybdenum cofactor in humans. Proc. Natl. Acad. Sci. USA **101:** 5946–5951
94 Nieder J., Stallmeyer B., Brinkmann H. and Mendel R. R. (1997) Identification of A. thaliana cDNAs homologous to the E. coli sulphotransferase MoeB. In: Sulphur Metabolism in Higher Plants, pp. 275–277, Cram W. J., De Kok L. J., Stulen I., Brunold C. and Rennenberg H. (eds.), Backhuys, Leiden
95 Matthies A., Nimtz M. and Leimkuhler S. (2005) Molybdenum cofactor biosynthesis in humans: identification of a persulfide group in the rhodanese-like domain of MOCS3 by mass spectrometry. Biochemistry **44:** 7912–7920
96 Grunden A. M. and Shanmugam K. T. (1997) Molybdate transport and regulation in bacteria. Arch. Microbiol. **168:** 345–354
97 Amy N. K. (1981) Identification of the molybdenum cofactor in chlorate-resistant mutants of Escherichia coli. J. Bacteriol.**148:** 274–282
98 Anderson L. A., Palmer T., Price N. C., Bornemann S., Boxer D. H. and Pau R. N. (1997) Characterisation of the molybdenum-responsive ModE regulatory protein and its binding to the promoter region of the modABCD (molybdenum transport) operon of Escherichia coli. Eur. J. Biochem. **246:** 119–126
99 Anderson L. A., McNairn E., Lubke T., Pau R. N. and Boxer D. H. (2000) ModE-dependent molybdate regulation of the molybdenum cofactor operon moa in Escherichia coli. J. Bacteriol. **182:** 7035–7043
100 Llamas A., Kalakoutskii K. L. and Fernandez E. (2000) Molybdenum cofactor amounts in *Chlamydomonas reinhardtii* depend on the *Nit5* gene function related to molybdate transport. Plant, Cell and Environment **23:** 1247–1255
101 Schwarz G., Schulze J., Bittner F., Eilers T., Kuper J., Bollmann G. et al. (2000) The molybdenum cofactor biosynthetic protein Cnx1 complements molybdate-repairable mutants, transfers molybdenum to the metal binding pterin, and is associated with the cytoskeleton. Plant Cell **12:** 2455–2472
102 Llamas A., Mendel R. R. and Schwarz G. (2004) Synthesis of adenylated molybdopterin: an essential step for molybdenum insertion. J. Biol. Chem. **279:** 55241–55246
103 Joshi M. S., Johnson J. L. and Rajagopalan K. V. (1996) Molybdenum cofactor biosynthesis in Escherichia coli mod and mog mutants. J. Bacteriol. **178:** 4310–4312.
104 Schwarz G., Boxer D. H. and Mendel R. R. (1997) Molybdenum cofactor biosynthesis The plant protein Cnx1 binds molybdopterin with high affinity. J. Biol. Chem. **272:** 26811–26814
105 Kuper J., Palmer T., Mendel R. R. and Schwarz G. (2000) Mutations in the molybdenum cofactor biosynthetic protein Cnx1G from Arabidopsis thaliana define functions for molybdopterin bind, Mo-insertion and molybdenum cofactor stabilization. Proc. Natl. Acad. Sci. USA **97:** 6475–6480
106 Schwarz G., Schrader N., Mendel R. R., Hecht H. J. and Schindelin H. (2001) Crystal structures of human gephyrin and plant Cnx1 G domains: comparative analysis and functional implications. J. Mol. Biol. **312:** 405–418
107 Kuper J., Winking J., Hecht H. J., Mendel R. R. and Schwarz G. (2003) The active site of the molybdenum cofactor biosynthetic protein domain Cnx1G. Arch. Biochem. Biophys. **411:** 36–46
108 Kuper J., Meyer zu Berstenhorst S., Vodisch B., Mendel R. R., Schwarz G. and Boxer D. H. (2003) In vivo detection of molybdate-binding proteins using a competition assay with ModE in Escherichia coli. FEMS Microbiol. Lett. **218:** 187–193
109 Hasona A., Ray R. M. and Shanmugam K. T. (1998) Physiological and genetic analyses leading to identification of a biochemical role for the moeA (molybdate metabolism) gene product in Escherichia coli. J. Bacteriol. **180:** 1466–1472
110 Kredich N. M. (1996) Biosynthesis of cysteine. In: Escherichia coli and Salmonella typhimurium: cellular and molecular biology, pp. 514–527, Neidhardt F. C., Curtiss R. III, Ingraham J. L., Lin E. C. C., Low K. B., Magasanik B. et al. (eds.), ASM Press, Washington, DC
111 Seubert P. A., Grant P. A., Christie E. A., Farley J. R. and Segel I. H. (1979) Kinetic and chemical properties of ATP sulphurylase from Penicillin chrysogenum. Ciba Found. Symp. 19–47
112 Bick J. A. and Leustek T. (1998) Plant sulfur metabolism – the reduction of sulfate to sulfite. Curr. Opin. Plant Biol. **1:** 240–244
113 Loschi L., Brokx S. J., Hills T. L., Zhang G., Bertero M. G., Lovering A. L. et al. (2004) Structural and biochemical identification of a novel bacterial oxidoreductase. J. Biol. Chem. **279:** 50391–50400
114 Leimkühler S., Angermüller S., Schwarz G., Mendel R. R. and Klipp W. (1999) Activity of the molybdopterin-containing xanthine dehydrogenase of Rhodobacter capsulatus can be re-

stored by high molybdenum concentrations in a moeA mutant defective in molybdenum cofactor biosynthesis. J. Bacteriol. **181:** 5930–5939
115 Nichols J. D. and Rajagopalan K. V. (2005) In vitro molybdenum ligation to molybdopterin using purified components. J. Biol. Chem. **280:** 7817–7822
116 Dobbek H., Gremer L., Meyer O. and Huber R. (1999) Crystal structure and mechanism of CO dehydrogenase, a molybdo iron-sulfur flavoprotein containing *S*-selanylcysteine. Proc. Natl. Acad. Sci. USA **96:** 8884–8889
117 Hille R. (2002) Molybdenum enzymes containing the pyranopterin cofactor: an overview. Met. Ions Biol. Syst. **39:** 187–226
118 Johnson J. L., Indermaur L. W. and Rajagopalan K. V. (1991) Molybdenum cofactor biosynthesis in Escherichia coli. Requirement of the chlB gene product for the formation of molybdopterin guanine dinucleotide. J. Biol. Chem. **266:** 12140–12145
119 Palmer T., Vasishta A., Whitty P. W. and Boxer D. H. (1994) Isolation of protein FA, a product of the mob locus required for molybdenum cofactor biosynthesis in Escherichia coli. Eur. J. Biochem. **222:** 687–692
120 Palmer T., Goodfellow I. P., Sockett R. E., McEwan A. G. and Boxer D. H. (1998) Characterisation of the mob locus from Rhodobacter sphaeroides required for molybdenum cofactor biosynthesis. Biochim. Biophys. Acta **1395:** 135–140
121 Eaves D. J., Palmer T. and Boxer D. H. (1997) The product of the molybdenum cofactor gene mobB of Escherichia coli is a GTP-binding protein. Eur. J. Biochem. **246:** 690–697
122 Blasco F., Dos Santos J. P., Magalon A., Frixon C., Guigliarelli B., Santini C. L. et al. (1998) NarJ is a specific chaperone required for molybdenum cofactor assembly in nitrate reductase A of Escherichia coli. Mol. Microbiol. **28:** 435–447
123 Hänzelmann P. and Meyer O. (1998) Effect of molybdate and tungstate on the biosynthesis of CO dehydrogenase and the molybdopterin cytosine-dinucleotide-type of molybdenum cofactor in Hydrogenophaga pseudoflava. Eur. J. Biochem. **255:** 755–765
124 Rothery R. A., Grant J. L. S., Johnson J. L., Rajagopalan K. V. and Weiner J. H. (1995) Association of molybdopterin guanine dinucleotide with *Escherichia coli* dimethyl sulfoxide reductase: effect of tungstate and a *mob* mutation. J. Bact. **177:** 2057–2063
125 Rothery R. A., Magalon A., Giordano G., Guigliarelli B., Blasco F. and Weiner J. H. (1998) The molybdenum cofactor of Escherichia coli nitrate reductase A (NarGHI). Effect of a mobAB mutation and interactions with [Fe-S] clusters. J. Biol. Chem. **273:** 7462–7469
126 Stevenson C. E. M., Sargent F., Buchanan G., Palmer T. and Lawson D. M. (2000) Crystal structure of the molybdenum cofactor biosynthesis protein MobA from *Escherichia coli* at near-atom resolution. Structure **8:** 1115–1125
127 Wahl R. C., Warner C. K., Finnerty V. and Rajagopalan K. V. (1982) Drosophila melanogaster ma-l mutants are defective in the sulfuration of desulfo Mo hydroxylases. J. Biol. Chem. **257:** 3958–3962
128 Scazzocchio C., Holl F. B. and Foguelman A. I. (1973) The genetic control of molybdoflavoproteins in Aspergillus nidulans. Allopurinol-resistant mutants constitutive for xanthine-dehydrogenase. Eur. J. Biochem. **36:** 428–445
129 Leon-Klooserziel K. M., Gil. M. A., Ruijs G. J., Jacobsen S. E., Olszewski N. E., Schwartz S. H. et al. (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at tow new loci. Plant J. **10:** 655–661
130 Ichida K., Matsumura T., Sakuma R., Hosoya T. and Nishino T. (2001) Mutation of human molybdenum cofactor sulfurase gene is responsible for classical xanthinuria type II. Biochem. Biophys. Res. Commun. **282:** 1194–1200
131 Heidenreich T., Wollers S., Mendel R. R. and Bittner F. (2005) Characterization of the NifS-like domain of ABA3 from Arabidopsis thaliana provides insight into the mechanism of molybdenum cofactor sulfuration. J. Biol. Chem. **280:** 4213–4218
132 Amrani L., Primus J., Glatigny A., Arcangeli L., Scazzocchio C. and Finnerty V. (2000) Comparison of the sequences of the Aspergillus nidulans hxB and Drosophila melanogaster ma-l genes with nifS from Azotobacter vinelandii suggests a mechanism for the insertion of the terminal sulphur atom in the molybdopterin cofactor. Mol. Microbiol. **38:** 114–125
133 Xiong L., Ishitani M., Lee H. and Zhu J. K. (2001) The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress- and osmotic stress-responsive gene expression. Plant Cell **13:** 2063–2083
134 Ketchum P. A. and Sevilla C. L. (1973) In vitro formation of nitrate reductase using extracts of the nitrate reductase mutant of Neurospora crassa, nit-1, and Rhodospirillum rubrum. J. Bacteriol. **116:** 600–609
135 Amy N. K. and Rajagopalan K. V. (1979) Characterization of molybdenum cofactor from Escherichia coli. J. Bacteriol. **140:** 114–124
136 Aguilar M., Cardenas J. and Fernandez E. (1992) Quantitation of molybdopterin oxidation product in wild-type and molybdenum cofactor deficient mutants of Chlamydomonas reinhardtii. Biochim. Biophys. Acta **1160:** 269–274
137 Ataya F. S., Witte C. P., Galvan A., Igeno M. I. and Fernandez E. (2003) Mcp1 Encodes the Molybdenum Cofactor Carrier Protein in Chlamydomonas reinhardtii and Participates in Protection, Binding, and Storage Functions of the Cofactor. J. Biol. Chem. **278:** 10885–10890
138 Leimkuhler S. and Rajagopalan K. V. (2001) In vitro incorporation of nascent molybdenum cofactor into human sulfite oxidase. J. Biol. Chem. **276:** 1837–1844
139 Johnson J. L. and Duran M. (2001) Molybdenum cofactor deficiency and isolated sulfite oxidase deficiency. In: The Metabolic and Molecular Bases of Inherited Disease, pp. 3163–3177, Scriver C., Beaudet A., Sly W., Valle D. (eds.), McGraw-Hill, New York
140 Reiss J. and Johnson J. L. (2003) Mutations in the molybdenum cofactor biosynthetic genes MOCS1, MOCS2, and GEPH. Hum. Mutat. **21:** 569–576
141 Langosch D., Hoch W. and Betz H. (1992) The 93 kDa protein gephyrin and tubulin associated with the inhibitory glycine receptor are phosphorylated by an endogenous protein kinase. FEBS Lett. **298:** 113–117
142 Kirsch J., Wolters I., Triller A. and Betz H. (1993) Gephyrin antisense oligonucleotides prevent glycine receptor clustering in spinal neurons [see comments]. Nature **366:** 745–748
143 Feng G., Tintrup H., Kirsch J., Nichol M. C., Kuhse J., Betz H. et al. (1998) Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity [see comments]. Science **282:** 1321–1324
144 Reiss J., Gross-Hardt S., Christensen E., Schmidt P., Mendel R. R. and Schwarz G. (2001) A mutation in the gene for the neurotransmitter receptor-clustering protein gephyrin causes a novel form of molybdenum cofactor deficiency. Am. J. Hum. Genet. **68:** 208–213
145 Duran M., Beemer F. A., van de Heiden C., Korteland J., de Bree P. K., Brink M. et al. (1978) Combined deficiency of xanthine oxidase and sulphite oxidase: a defect of molybdenum metabolism or transport? J. Inherit. Metab. Dis. **1:** 175–178
146 Johnson J. L. and Rajagopalan K. V. (1995) An HPLC assay for detection of elevated urinary S-sulphocysteine, a metabolic marker of sulphite oxidase deficiency. J. Inherit. Metab. Dis. **18:** 40–47
147 Johnson J. L., Wuebbens M. M., Mandell R. and Shih V. E. (1989) Molybdenum cofactor biosynthesis in humans. Identification of two complementation groups of cofactor-deficient patients and preliminary characterization of a diffusible molybdopterin precursor. J. Clin. Invest. **83:** 897–903

148 Reiss J., Christensen E. and Dorche C. (1999) Molybdenum cofactor deficiency: first prenatal genetic analysis. Prenat. Diagn. **19:** 386–388
149 Lee H.-J., Adham I. M., Schwarz G., Kneussel M., Sass J.-O., Engel W. et al. (2002) Molybdenum cofactor-deficient mice resemble the phenotype of human patients. Hum. Mol. Gen. **11:** 3309–3317
150 Blau N., Thöny B., Cotton R. G. H. and Hyland K. (2001) Disorders of tetrahydrobiopterin and related biogenic amines. In: The Metabolic and Molecular Bases of Inherited Disease, pp. 1725–1776, Scriver C., Beaudet A., Sly W. and Valle D. (eds), McGraw-Hill, New York
151 Kramer S., Hageman R. V. and Rajagopalan K. V. (1984) In vitro reconstitution of nitrate reductase activity of the Neurospora crassa mutant nit-1: specific incorporation of molybdopterin. Arch. Biochem. Biophys. **233:** 821–829
152 Schwarz G., Santamaria-Araujo J. A., Wolf S., Lee H. J., Adham I. M., Grone H. J. et al. (2004) Rescue of lethal molybdenum cofactor deficiency by a biosynthetic precursor from Escherichia coli. Hum. Mol. Gen. **13:** 1249–1255
153 Johnson J. L. and Rajagopalan K. V. (1993) Molybdopterin biosynthesis in man. Properties of the converting factor in liver tissue from a molybdenum cofactor deficient patient. Adv. Exp. Med. Biol. **338:** 379–382
154 Esnouf R. M. (1997) An extensively modified version of MolScript that includes greatly enhanced coloring capabilities. J. Mol. Graph. Model. **15:** 132–134, 112–133
155 Rajagopalan K. V. (1996) Biosynthesis of the molybdenum cofactor. In: Escherichia coli and Salmonella typhimurium, pp. 674–679, Neidhardt F. C. (ed.), ASM Press, Washington DC
156 GeneBank cnx7accession number AF208343
157 GeneBank cnx6accession number AJ133519
158 Stallmeyer B., Coyne K. E., Wuebbens M., Johnson J. L., Rajagopalan K. V. and Mendel R. R. (1998) mocs3. GeneBank AF102544
159 Bader G., Gomez-Ortiz M., Haussmann C., Bacher A., Huber R. and Fischer M. (2004) Structure of the molybdenum-cofactor biosynthesis protein MoaB of Escherichia coli. Acta Crystallogr. D Biol. Crystallogr. **60:** 1068–1075
160 Stallmeyer B., Nerlich A., Schiemann J., Brinkmann H. and Mendel R. R. (1995) Molybdenum co-factor biosynthesis: the Arabidopsis thaliana cDNA cnx1 encodes a multifunctional two-domain protein homologous to a mammalian neuroprotein, the insect protein Cinnamon and three Escherichia coli proteins. Plant J. **8:** 751–762
161 Sola M., Bavro V. N., Timmins J., Franz T., Ricard-Blum S., Schoehn G. et al. (2004) Structural basis of dynamic glycine receptor clustering by gephyrin. EMBO J. **23:** 2510–2519
162 Palmer T., Santini C.-L., Iobbi-Nivol C., Eaves D. J., Boxer D. H. and Giordano G. (1996) Involvement of the *narJ* and *mob* gene products in distinct steps in the biosynthesis of the molybdoenzyme nitrate reductase in *Escherichia coli*. Mol. Microbiol. **20:** 875–884